

# UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.  
FG0810

Total Pages in this Submission

**TO THE ASSISTANT COMMISSIONER FOR PATENTS**Box Patent Application  
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

**METHODS FOR DETECTING, PREVENTING, AND TREATING RENAL DISORDERS BY MODULATING, REGULATING, AND INHIBITING CONNECTIVE TISSUE GROWTH FACTOR**

and invented by:

Bruce L. Riser  
Mark DeNichilo

If a CONTINUATION APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No.: 60/099,471

Which is a:

And 60/112,855

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Which is a:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Enclosed are:

**Application Elements**

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having \_\_\_\_\_ pages and including the following:
  - a. ☒ Descriptive Title of the Invention
  - b. ☒ Cross References to Related Applications (if applicable)
  - c. ☐ Statement Regarding Federally-sponsored Research/Development (if applicable)
  - d. ☐ Reference to Microfiche Appendix (if applicable)
  - e. ☒ Background of the Invention
  - f. ☒ Brief Summary of the Invention
  - g. ☒ Brief Description of the Drawings (if drawings filed)
  - h. ☒ Detailed Description
  - i. ☒ Claim(s) as Classified Below
  - j. ☒ Abstract of the Disclosure

# UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.  
FG0810

Total Pages in this Submission

## Application Elements (Continued)

3. ☒ Drawing(s) (when necessary as prescribed by 35 USC 113)
- a. ☐ Formal      b. ☒ Informal      Number of Sheets \_\_\_\_\_
4. ☒ Oath or Declaration
- a. ☐ Newly executed (original or copy)      ☒ Unexecuted
- b. ☐ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional application only)
- c. ☐ With Power of Attorney      ☐ Without Power of Attorney
- d. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in the prior application,  
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (usable if Box 4b is checked)  
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche
7. ☐ Genetic Sequence Submission (if applicable, all must be included)
- a. ☐ Paper Copy
- b. ☐ Computer Readable Copy
- c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

## Accompanying Application Parts

8. ☐ Assignment Papers (cover sheet & documents)
9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement/PTO-1449      ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☐ Acknowledgment postcard
14. ☒ Certificate of Mailing
- ☐ First Class      ☒ Express Mail (Specify Label No.): EL278506943US

# UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.  
FG0810

Total Pages in this Submission

## Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)

16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: \_\_\_\_\_

17. ☐ Additional Enclosures (please identify below):

## Fee Calculation and Transmittal

### CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	18	- 20 =	0	x \$11.00	\$0.00
Indep. Claims	6	- 3 =	3	x \$41.00	\$123.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$395.00
OTHER FEE (specify purpose) _____					\$0.00
TOTAL FILING FEE					\$518.00

- ☐ A check in the amount of \_\_\_\_\_ to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **50-0811** as described below. A duplicate copy of this sheet is enclosed.
- ☒ Charge the amount of **\$518.00** as filing fee.
  - ☒ Credit any overpayment.
  - ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
  - ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: September 8, 1999

  
Signature

Mariette A. Lapiz  
Reg. No. 44,202

CC:

**APPLICATION**

**in**

**THE UNITED STATES  
PATENT AND TRADEMARK OFFICE**

**for**

**METHODS FOR DETECTING, PREVENTING, AND TREATING  
RENAL DISORDERS BY MODULATING, REGULATING, AND INHIBITING  
CONNECTIVE TISSUE GROWTH FACTOR**

**Inventors:**

**Bruce L. Riser and Mark DeNichilo**

**FibroGen, Inc.**

**225 Gateway Boulevard**

**South San Francisco, California 94080**

**(650) 866-7200**

**and**

**Henry Ford Hospital and Medical Center**

**2799 West Grand Boulevard CFP-5**

**Detroit, Michigan 48202**

**(313) 876-2710**

**Attorney Reference No.: FG0810**

652060-722660

**METHOD FOR DETECTING, PREVENTING, AND TREATING  
RENAL DISORDERS BY MODULATING, REGULATING, AND INHIBITING  
CONNECTIVE TISSUE GROWTH FACTOR**

Inventors: Bruce L. Riser and Mark DeNichilo

**I. FIELD OF THE INVENTION**

The present invention relates to the role of Connective Tissue Growth Factor (CTGF) in the production of extracellular matrix. More specifically, the invention relates to methods of detecting, preventing, and treating kidney fibrosis and other conditions associated with overproduction of the extracellular matrix by targeting CTGF.

**II. BACKGROUND OF THE INVENTION**

***Kidney Diseases And Disorders.*** The kidney functions to separate waste products from the blood, regulate acid concentration, and maintain water balance. Kidneys control the levels of various compounds in the blood, such as hydrogen, sodium, potassium, and silicon, and eliminate waste in the form of urine. Any degradation in kidney function can interfere with the body's ability to adequately remove metabolic products from the blood, and can disrupt the body's electrolyte balance. In its most severe forms, degradation or impairment of kidney function can be fatal.

A number of conditions can lead to chronic renal failure, a decline in kidney function over time. For example, such conditions as hypertension, diabetes, congestive heart failure, lupus, and sickle cell anemia have been associated with renal failure. Acute disease processes and injuries can trigger a more immediate decline in kidney function.

It is thus well understood that individuals with diabetes, hypertension, inflammatory and autoimmune diseases, and other disorders are at risk for altered and progressive loss of kidney function characterized by, for example, reduced glomerular filtration, albuminuria, proteinuria,

5 and progressive renal insufficiency. More than half of the total number of kidney disorders initiate kidney fibrosis. Fibrosis involves altered formation or production of fibrous tissue, and can result in the overproduction and increased deposition of extracellular matrix components.

The extracellular matrix (ECM) is a complex network of various glycoproteins, polysaccharides, and other macromolecules secreted from a cell into extracellular space. The ECM provides a supportive framework, directly influencing various cellular characteristics, including shape, motility, strength, flexibility, and adhesion. In fibrosis, overproduction and increased deposition of ECM materials can result in thickening and malformation of various membranous and cellular components, reducing local flexibility and surface area of the affected site, and impairing a number of bodily processes.

15 Kidney fibrosis is a common pathway in the progression of various forms of renal injury. Kidney fibrosis typically spreads by enlisting previously undamaged regions of the kidney. As normal filtration processes decline, function of surviving tissue and of various regions of the kidney is systematically destroyed. Kidney fibrosis can be manifested as a diffuse thickening of kidney membranous components, the accumulation and expansion leading to a loss of filtration surface area and a corresponding disruption in the body's electrolyte composition and acid-base balance.

Fibrosis of the kidney is observed in a number of conditions, including, for example, diabetic, autoimmune, and transplant nephropathy; hypertension; and certain forms of glomerular injury or disease. Diabetes mellitus (diabetes) is a complex disease that affects several hundred million people worldwide. Diabetes is characterized by hyperglycemia or elevated levels of glucose in the blood. Glucose cannot enter the body's cells to be utilized and therefore remains in the blood in high concentrations. When the blood glucose level exceeds the reabsorptive capacity of the renal tubules, glucose is excreted in the urine. Diabetes produces a number of debilitating and life-threatening complications.

30 Progressive nephropathy is one of the most frequent and serious complications of diabetes. See, e.g., Hans-Henrik *et al.*, 1988, *Diabetic Nephropathy: The Second World Conference on Diabetes Research, New Frontiers. The Juvenile Diabetes Foundation*

5 *International*, pp. 28-33. A hallmark of diabetic nephropathy, and of renal sclerosis due to other forms of renal injury, is early expansion of the glomerular mesangium, largely due to increased accumulation of ECM proteins such as collagen types I and IV, fibronectin, and laminin. See, e.g., Mauer *et al.*, 1984, *J. Clin. Invest.* 74:1143-1155; Bruneval *et al.*, 1985, *Human Pathol.* 16:477-484. This pathological deposition results in impaired filtration, leading to renal failure, a condition requiring transplantation or life-long dialysis. Current therapies slow but do not arrest or reverse the progressive loss of kidney function. Predominant causal factors identified to date also include hyperglycemia, glomerular hypertension, and abnormal cytokine environments. Tuttle, *et al.*, 1991, *N. Engl. J. Med.* 324:1626-1632; The Diabetes Control Complications Trial Research Group, 1993, *N. Engl. J. Med.* 329:977-986; Hostetter *et al.*, 1981, *Kidney Int.* 19:410-415; Anderson *et al.*, 1985, *J. Clin. Invest.* 76:612-619; Border *et al.*, 1993, *Am J. Kidney Dis.* 22:105-113.

Hyperglycemia may be damaging, in great part as increased concentrations of glucose stimulate ECM accumulation by mesangial cells. See, e.g., Ayo *et al.*, 1990, *Am. J. Pathol.* 136:1339-1348; Heneda *et al.*, 1991, *Diabetologia* 34:190-200; Nahman *et al.*, 1992, *Kidney Int* 41:396-402; Cortes *et al.*, 1997, *Kidney Int.* 51:57-68. As shown by Davies *et al.*, 1992, *Kidney Intl.* 41:671-678, mesangial cells are largely responsible for mesangial matrix synthesis *in situ*. It has further been determined that the effect of glucose on mesangial cell matrix production is linked to increased glucose transport and utilization. Helig *et al.*, 1995, *J. Clin. Invest.* 96:1802-1814. Moreover, Ziyadeh *et al.*, 1994, *J. Clin Invest.* 93:536-542, have shown the involvement of secreted soluble mediators on mesangial cell matrix production.

Renal hypertension, which can appear as a secondary manifestation of kidney disease in diabetic patients, can also result from other diseases or disorders, including long-standing hypertension. Secondary hypertension can be caused by virtually any impairment in renal function. A greater understanding of the pathogenic mechanisms for hypertension-induced ECM deposition is developing. For example, in diabetes, an early impairment of normal blood pressure dampening occurs at the glomerular afferent arteriole, resulting in the exposure of glomerular capillaries to large moment-to-moment variations in systemic blood pressure.

Hayashi *et al.*, 1992, *J. Am. Soc. Nephrol.* 2:1578-1586; Bidani *et al.*, 1993, *Am. J. Physiol.* 265:F391-F398. Due to the elasticity of the glomerulus, increased capillary pressure produces expansion of glomerular structure, resulting in augmentation of the mechanical strain imposed on the mesangial cells. Riser *et al.*, 1992, *J. Clin. Invest.* 90:1932-1943; Kriz *et al.*, *Kidney Int.* 38 (Suppl. 20):S2-S9. In addition, when cultured mesangial cells are subjected to cyclic strain, the mesangial cells respond by increasing the synthesis and accumulation of collagen types I and IV, fibronectin, and laminin. Riser *et al.*, 1992, *supra*. While increased glomerular pressure is common in diabetes, it is not limited to this disease, and is present in other forms of progressive renal disorders, including, for example, certain forms of glomerular nephritis and hypertrophy. See, e.g., Cortes *et al.*, 1997, *Kidney Intl.* 51:57-68.

Kidney fibrosis and associated renal impairment are thus present in the progression of various diseases and disorders, including diabetes and hypertension, and methods of treating kidney fibrosis are thus greatly desired.

**Transforming Growth Factor  $\beta$  (TGF- $\beta$ ).** The few studies conducted to date regarding the physiological implications of renal disorders and diseases, and, in particular, those due to diabetes, have focused on the role of transforming growth factor- $\beta$  (TGF- $\beta$ ) in developing methods for targeting overproduction (increased synthesis and accumulation) of extracellular matrix components. The role of cytokine imbalance in initiating and/or perpetuating glomerular matrix expansion has been explored in experimental nephropathy studies involving TGF- $\beta$ . See, e.g., Sharma *et al.*, *Seminars In Nephrology* 1:116-129. Glomerular TGF- $\beta$  activity is increased in both human and experimental diabetic glomerulosclerosis. See, e.g., Yamamoto *et al.*, 1993, *Proc Natl Acad Sci* 90:1814-1818; Sharma *et al.*, 1994, *Am J Physiol* 267:F1094-F1101; Shankland *et al.*, 1994, *Kidney Int* 46:430-442. The exposure of cultured mesangial cells or glomeruli to TGF- $\beta$  results in increased ECM production. See, e.g., Bollineni *et al.*, 1993, *Diabetes* 42:1673-1677. *In vivo* induction of glomerular matrix accumulation following transfection and overexpression of the TGF- $\beta$  gene in rat kidney has been demonstrated by, for example, Isaka *et al.*, *J. Clin. Invest* 92:2597-2601.



5 In addition, neutralization studies have shown that anti-TGF- $\beta$  antibody mitigates the enhanced glomerular ECM gene expression that occurs in experimental glomerulonephritis and diabetes. Border *et al.*, 1990, *Nature* 346:371-374; Sharma *et al.*, 1996, *Diabetes* 45:522-530. The sustained overexpression of glomerular TGF- $\beta$  in diabetes may be the result of a mesangial cellular response to both increased glucose levels and hypertension. It has been reported that exposure of mesangial cells to increased concentrations of glucose in the medium stimulates the synthesis and release of TGF- $\beta$ 1, as well as the increased binding of TGF- $\beta$  to specific receptors. Ziyadeh *et al.*, 1994, *J. Clin Invest* 93:536-542; Riser *et al.*, 1998, *J. Am. Soc. Nephrol.* 9:827-836; Riser *et al.*, 1999, *Kidney Int.*, 56:428-439. It has also been reported that mechanical force selectively stimulates the production, release, and activation of TGF- $\beta$ 1, as well as the increased expression of TGF- $\beta$  receptors. Riser *et al.*, 1996, *Am. J. Path.* 148:1915-1923.

*In vitro* neutralization studies of TGF- $\beta$  demonstrated a significant reduction of collagen synthesis induced in mesangial cells by increased glucose levels. See, e.g., Sharma *et al.*, 1996, *supra*; Ziyadeh *et al.*, 1994, *supra*. Studies have also shown a virtual elimination of collagen accumulation resulting from cyclic stretching in the presence of excess glucose. Riser *et al.*, 1997, *supra*. TGF- $\beta$  stimulates the proliferation of mesangial cells *in vitro* and *in vivo*, and may induce in these replicating cells overproduction and increased deposition of ECM characteristic of various renal disorders, including proliferative disorders such as glomerular nephritis. See, e.g., Border, W.A. *et al.*, 1990, *Nature* 346:371-374; Habershroh, U.G., *et al.*, 1993, *Am. J. Physiol.* 264:F199-205. As a result of these findings, intense efforts have been directed toward reducing TGF- $\beta$  availability and binding as a means of mitigating matrix accumulation. However, the ubiquitous nature and pluripotent functions of TGF- $\beta$ , including tumor suppression and the multiple levels of regulation, raise questions concerning both the feasibility and the safety of its long-term inhibition. See, e.g., Brattain *et al.*, 1996, *Curr. Opin. Oncol.*:49-53; Franklin, 1997, *Int. J. Biochem. Cell Biol.* 29:79-89.

Therefore, a method for treating or preventing ECM overproduction or increased deposition, without interfering with the ubiquitous function of TGF- $\beta$ , is needed.

**Connective Tissue Growth Factor (CTGF).** CTGF is a peptide that may act downstream of TGF- $\beta$  to regulate matrix accumulation. This novel growth factor has been reported and described previously. *See, e.g.,* U.S. Patent No. 5,408,040; Bradham *et al.*, 1991, *J. Cell Biology* 114:1285-1294. CTGF is characterized as a polypeptide which exists as a monomer with a molecular weight of approximately 36 to 38 kD. CTGF has been shown to be one of seven cysteine-rich secreted proteins belonging to the CCN family, which includes CTGF, cyr-61, and nov. Oemar *et al.*, 1997, *Arteriosclerosis, Thrombosis and Vascular Biology* 17(8):1483-1489. CTGF is an immediate early response gene that codes for a protein consisting of four modules and one signal peptide. Oemar *et al.*, 1997, *supra*. The four modules are: 1) an insulin-like growth factor (IGF) binding domain, 2) a von Willebrand factor type C repeat most likely involved in oligomerization, 3) a thrombospondin type 1 repeat believed to be involved in binding to the ECM, and 4) a C-terminal module which may be involved in receptor binding. Recent reports suggest that certain fragments of the whole CTGF protein possess CTGF activity. *See, e.g.,* Brigstock, *et al.*, 1997, *J. Bio. Chem.* 272(32):20275-282. Human, mouse, and rat CTGF are highly conserved with greater than 90% amino acid homology and a molecular weight of about 38 kD. It was recently shown that the promoter of CTGF contains a novel TGF- $\beta$  responsive element. Grotendorst *et al.*, 1996, *Cell Growth & Differentiation* 7:469-480.

It appears that CTGF may be an important prosclerotic molecule in both skin fibrosis and cardiac atherosclerosis. For example, CTGF mRNA is expressed by fibroblasts in the lesions of patients with systemic sclerosis, keloids, and localized scleroderma, while there is no corresponding expression in adjacent normal skin. *See, e.g.,* Igarashi *et al.*, 1995, *The Journal of Investigative Dermatology* 105:280-284; Igarashi *et al.*, 1996, *The Journal of Investigative Dermatology* 106:729-733. Cultured normal human skin fibroblasts respond to TGF- $\beta$  but not to platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or basic fibroblast growth factor (bFGF), by increasing levels of CTGF mRNA and CTGF protein. Igarashi *et al.*, *Molecular Biology of the Cell* 4:637-645. Fibroblasts from lesions of scleroderma show increased mitogenesis to TGF- $\beta$  and produce greater amounts of CTGF than do normal fibroblasts. Kikuche *et al.*, 1995, *Journal of Invest. Dermatology* 105:128-132. Recombinant

human CTGF injected under the skin of NIH Swiss mice induces the same rapid and dramatic increase in connective tissue cells and ECM as occurs with TGF- $\beta$  treatment, whereas PDGF and EGF have little or no effect on granulation. Frazier *et al.*, 1996, *The Journal of Investigative Dermatology* 107:404-411. Cultured vascular smooth muscle cells are also stimulated by TGF- $\beta$  to produce CTGF. In heart disease patients, CTGF mRNA is expressed at levels 50- to 100-fold higher in atherosclerotic plaques than in normal arteries. Oemar *et al.*, 1997, *Circulation* 95(4):831-839.

In spite of mounting evidence implicating CTGF as a causal factor in skin fibrosis and cardiac atherosclerosis, very little is known of its expression in, for example, renal sclerosis or diabetes. It has been shown, using an *in vitro* model of calcium oxalate nephrolithiasis, that monkey kidney epithelial cells respond to calcium oxalate by upregulating the CTGF gene along with other genes involved in matrix turnover. Hammes *et al.*, 1995, *Kidney International* 48:501-509. A similar response occurs in cultured renal epithelial cells following mechanical wounding. See, e.g., Pawar *et al.*, 1995, *Journal of Cellular Physiology* 165:556-565. Most recently, CTGF mRNA was found in biopsies from normal human kidneys. A qualitative assessment indicated that, in a limited number of cases, CTGF expression was increased in the tissues of patients with severe mesangial proliferative lesions of crescentic glomerulonephritis, focal and segmented glomerulosclerosis, and, in three cases, diabetic glomerulosclerosis. Ito *et al.*, 1998, *Kidney International* 53:853-861. The research, relying only on data obtained from biopsies, did not include quantitative results or any measurement of CTGF protein levels.

Further, no connection between CTGF mRNA levels and the production and deposition of ECM, and no quantitative method for detecting renal disorders or diseases, including diabetes, involving a determination of CTGF levels in samples, and did not identify CTGF-expressing cells.

The role of CTGF in kidney diseases is thus unclear, and there has been no research to date has shown that CTGF is causally related to ECM overproduction and increased deposition and to fibrosis in the kidney.

5           **Diagnostics and Early-Stage Detection.** Kidney failure is a serious condition requiring extreme treatment such as hemodialysis or transplantation. Early-stage detection and/or prevention of any deviation from normal kidney pathology and function could minimize the risk of a subject's developing a more serious condition. Hypertension, for example, might be undetectable by a patient in early stages, but can be deadly if not identified, monitored, and  
10 treated. In addition, in some diseases, such as, for example, diabetes, less invasive and disruptive and more affordable means of treatment, such as dietary modification, are effective only at early stages. Therefore, there is a critical need for effective and reliable methods of diagnosis that permit early stage detection, and corresponding prevention, of renal complications.

For example, kidney failure resulting from progressive glomerulosclerosis is the leading  
15 cause of morbidity and mortality among patients with type I, or juvenile, diabetes mellitus. *See, e.g.,* Dorman, J.S. *et al.*, 1984, *Diabetes* 33:271-276; Anderson, A.R. *et al.*, 1983, *Diabetologia* 25:496-501. Current therapy with angiotensin-converting enzyme (ACE) inhibitors, the drug class of choice, effectively slows the progression of disease. *See, e.g.,* Lewis, E.J. *et al.*, 1993, *N. Eng. J. Med.*, 329:1456-1462. Nevertheless, this treatment is not justified in all newly diagnosed  
20 diabetic patients because only approximately 30-35% of these develop progressive kidney disease, and the long-term side effects of these drugs are uncertain. *See, e.g.,* Parving, H.-H. and E. Hommel, 1989, *Brit. Med. J.* 299:230-233. In addition, ACE inhibitors are also presently used to treat patients with hypertensive renal failure, including that resulting from non-diabetic nephropathies. However, the mechanism of renal protection, and, as noted above, the long-term  
25 side effects of this treatment are not fully understood. Furthermore, ACE inhibitors have been shown to negatively interact with nonsteroidal anti-inflammatory drugs. *See, e.g.,* Whelton, A., 1999, *Am. J. Med.* 106(SB):13S-24S.

In a current method of diagnosis, diabetic patients are monitored for microalbuminuria. Persistent microalbuminuria is a marker of widespread vascular damage and indicates the  
30 presence of early nephropathy in type 1 and type 2 diabetes. *See, e.g.,* Stehouwer, C.D. *et al.*, 1992, *Lancet* 340:319-323; Bojestig, M. *et al.*, 1996, *Diabetes Care* 19:313-317; Mogensen, C.E. *et al.*, 1995, *Lancet* 346:1080-1084. However, the actual level of microalbuminuria may not

necessarily predict the development of overt nephropathy, particularly among patients with a long duration of diabetes. Bojestig *et al.*, *supra*. In addition, since by the time microalbuminuria is detected, structural renal lesions are already present, the effectiveness of treatment to slow progression may be substantially reduced. Bangstad, H.-J. *et al.*, 1993, *Diabetologia* 36:523-529; Ruggenenti, P. *et al.*, 1998, *J. Am. Soc. Nephrol.* 9:2157-2169; Fioretto, P. *et al.*, 1995, *Kidney Int.* 48:1929-1935. There is a great need to be able to predict which patients with type 1 diabetes will develop nephropathy, and to, in general, develop a method that will detect renal alterations that may precede the onset of significant disease.

In summary, there is a need in the art for effective methods for diagnosing, treating, and preventing fibrosis associated with impairment and degradation of kidney function in a variety of diseases and disorders, most particularly, in diabetes and hypertension. No current research has focused on the modulation of CTGF expression or activity as a means of preventing or treating kidney fibrosis.

### III. SUMMARY OF THE INVENTION

The present invention satisfies the need in the art by providing methods for detecting, treating, and preventing renal disorders and diseases associated with fibrosis. In particular, the present invention provides methods for detecting, preventing, and treating pathologies and complications associated with renal disorders and conditions which are characterized by an overproduction or increased deposition of extracellular matrix.

***Methods Of Treatment and Prevention.*** The present invention provides various approaches directed to modulation of the overproduction of the extracellular matrix resulting in fibrosis. Specifically, the present invention provides methods of regulating increased accumulation of the extracellular matrix associated with kidney fibrosis as found in various renal diseases and disorders. These renal diseases and disorders include, but are not limited to, all kinds of nephropathy, including glomerulonephritis, glomerulosclerosis, and conditions resulting from glomerular injury; diabetic nephropathy and other complications; nephritis; interstitial disease; acute and chronic transplant rejection; renal hypertension, including that associated with

5 diabetes; and other underlying causes of fibrosis. More specifically, the present invention  
provides methods for preventing and treating complications associated with the above-named  
renal disease and disorders by regulating, modulating, and/or inhibiting the expression and  
activity of CTGF. In particular embodiments, the present methods are directed to the diagnosis,  
prevention, and treatment of renal diseases and disorders associated with diabetes or with  
10 hypertension.

The methods of the present invention provide for the administration of a therapeutically  
effective amount of an agent that regulates, modulates, and/or inhibits the ECM-producing  
activity of CTGF. In particular, the methods of this invention are useful for the treatment and  
prevention of renal disorders in mammals, most preferably, in humans.

15 In one aspect, the invention provides a method of treating complications associated with  
diabetes characterized by the overproduction or overaccumulation of the extracellular matrix by  
administering a therapeutically effective amount of an antibody reactive with a CTGF  
polypeptide or fragments thereof, or an antigen-binding fragment of an antibody reactive with the  
CTGF polypeptide or fragments thereof.

20 In another aspect, the present invention provides a method for treating and preventing  
complications associated with renal disorders, particularly, diabetes and hypertension, wherein  
antisense oligonucleotides which specifically bind to CTGF mRNA are used to interrupt  
expression of the protein product. The antisense oligonucleotides have a sequence capable of  
binding specifically with any polynucleotide sequences encoding CTGF or fragments thereof.

25 In yet a further embodiment of the present invention, a method is provided in which small  
molecules are used to inhibit the activity of CTGF or its active fragments by blocking the  
binding of CTGF to its receptor, inhibiting CTGF activity and thus thereby reducing the  
overproduction of the extracellular matrix associated with the onset and/or progression of renal  
disorders, including diabetes and hypertension.

30 The present invention further provides a method of treating and preventing renal  
disorders by administering a compound that blocks the binding interactions of or the enzymes  
involved in the signal transduction pathway of CTGF.

5 The present invention also provides a method for treating and preventing diabetes by administering insulin and an agent that modulates and/or inhibits the activity of CTGF. More specifically, the present invention discloses a method for treating and preventing diabetes by administering insulin and an agent that modulates, regulates, and/or inhibits the activity of CTGF according to the methods of the present invention.

10 Methods for evaluating the effectiveness of anti-fibrotic therapy, including the use of ACE inhibitors, by measuring the levels of CTGF in a sample from a subject undergoing a course of treatment for diseases and disorders associated with fibrosis, are also provided.

**Diagnostic Methods.** The present invention is also directed to methods for predicting which patients with diseases and disorders associated with renal disorders will subsequently develop progressive kidney disease. In one embodiment, the invention provides a method for detecting and/or staging (classifying the level, site, and spread of disease) kidney involvement in a particular disease or disorder. In one embodiment, a method of predicting whether a patient with diabetes will go on to develop progressive kidney disease is provided, along with a method of detecting the current level of kidney involvement, for example, in a subject with diabetes as opposed to a subject without diabetes.

The present invention is also directed to methods of detecting the presence of pathology of a tissue characterized by an excessive accumulation of extracellular matrix components. In one embodiment, the method involves determining the levels of CTGF, for example, through tissue biopsy or through non-intrusive methods, such as, for example, collection of a urine sample. In a particular embodiment, the method comprises determining the levels of CTGF in a sample comprising, for example, urine or other bodily fluids from a subject with a diabetic nephropathy, such as, for example, diabetic glomerulosclerosis. The method can also comprise determining the levels of CTGF in persons with progressive sclerosis, both with and without diabetes, by determining the levels of CTGF in urine or in other bodily fluids.

30 More specifically, the present invention comprises a means of diagnosing the presence of or a predisposition to kidney diseases and disorders, including a means for detecting and monitoring the pathogenesis of these diseases and disorders, or for detecting and monitoring the

5 presence of markers for the pathogenesis of these diseases and disorders. More specifically, the present invention provides for diagnosing renal disorders by measuring the levels of CTGF in a patient sample, preferably, in a urine sample from a patient.

In one embodiment of the present invention, a method is provided for the measurement of CTGF levels in a sample from a patient with no known or with a suspected renal disorder.

10 Comparison of CTGF levels in urine samples from a patient known to have a kidney disease or disorder, or from a patient known not to have any kidney disease or disorder, with CTGF levels in urine samples from a patient with no known or with a suspected kidney disorder can be indicative of the presence of a kidney disease or disorder. In particular, the method provides that higher levels of CTGF are present in samples from patients having renal disorders than in  
15 samples from patients without any renal disorder. Higher levels of CTGF are thus indicative of the presence of a disease or disorder associated with kidney fibrosis.

In another embodiment, the levels of CTGF can be measured by detecting CTGF mRNA or protein in a sample. In a further embodiment, the sample is a tissue sample and the presence of CTGF is detected by staining of the protein in the tissue or by determination of CTGF mRNA  
20 levels.

A preferred method of the present invention utilizes an antibody, preferably, a monoclonal antibody, capable of specifically binding to CTGF or active fragments thereof. The method of utilizing an antibody to measure the levels of CTGF allows for non-invasive diagnosis of the pathological states of kidney diseases. In a preferred embodiment of the present invention,  
25 the antibody is human or is humanized. The preferred antibodies may be used, for example, in standard radioimmunoassays or enzyme-linked immunosorbent assays or other assays which utilize antibodies for measurement of levels of CTGF in sample. In a particular embodiment, the antibodies of the present invention are used to detect and to measure the levels of CTGF present in a urine sample.

30 **Diagnostic Kit.** The present invention is further directed to diagnostic kits for detecting and measuring the levels of CTGF in a sample in order to detect a renal disorder or a predisposition to a renal disorder in a subject. In one embodiment, the kit contains antibodies



5 specific for CTGF and reagents for detecting and measuring CTGF in a sample. The sample can be a bodily fluid, such as urine, or, for example, a tissue sample. In one embodiment of the present invention, the kit comprises an immobilized antibody which specifically recognizes CTGF and an antibody specific for CTGF and capable of binding to an antigen component different from the immobilized antibody. The CTGF antibody can be enzyme-labeled, radio-  
10 labeled, or fluorescein-labeled. The kit can also comprises reagents necessary for detection of the antibody, and can further comprise other reagents as desired, such as, for example, dissolving agents, cleaning agents, and reaction terminators.

In a preferred embodiment of the invention, the kit is packaged, for example, in a box or a container which includes the necessary elements of the kit, and also includes instructions relating  
15 to the use of the kit.

#### IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and Figure 1B depict the effects of exogenous CTGF on mesangial cell secretion of the extracellular matrix. Figure 1A shows the quantities of fibronectin contained in the media at the end of incubation determined by ELISA. Figure 1B shows the quantities of collagen type I contained in the media at the end of incubation determined by ELISA.  
20

Figure 2 depicts CTGF gene expression in rat tissues and cultured kidney cells. Total RNA was extracted from whole organs of rat and from cultured rat mesangial cells and kidney fibroblasts for Northern analysis. MC represents mesangial cells; BT represents brain tissue; HT represents heart tissue; KT represents kidney tissue; and KFC represents kidney fibroblast cells.  
25

Figure 3A, Figure 3B, and Figure 3C depict regulation of CTGF and TGF- $\beta$  mRNA levels by exogenous TGF- $\beta$  and CTGF. Figure 3A shows results from a representative experiment. Figure 3B shows quantitation of mRNA bands for CTGF. Figure 3C shows quantitation of mRNA bands for TGF- $\beta$ .

Figure 4A and Figure 4B depict expression of CTGF protein by culture mesangial cells in the presence of exogenous TGF- $\beta$ . Figure 4A shows immunoblotting using an antibody raised  
30

against full length CTGF. Figure 4B shows immunoblotting using an antibody raised against a 15 amino acid sequence specific to CTGF.

Figure 5A and Figure 5B depict secretion of CTGF protein into the medium of mesangial cell cultures, and the effect of heparin. Figure 5A shows data relating to media pooled and heparin-sepharose treated for immunoblotting. Figure 5B shows data relating to media tested for CTGF content individually by ELISA prior to pooling.

Figure 6A and Figure 6B depict CTGF protein induction by mesangial cells. Figure 6A shows data relating to media pooled and heparin-sepharose treated for immunoblotting. Figure 6A shows data relating to media tested for CTGF content individually by ELISA prior to pooling.

Figure 7 depicts the effect of high glucose concentration on mesangial cells expression of CTGF mRNA. Figure 7 shows samples of pooled RNA from 6 different 100mm culture dishes in a representative experiment.

Figure 8 depicts TGF- $\beta$  blockade of high glucose-induced CTGF production using anti-TGF- $\beta$  antibody.

Figures 9A and 9B depict CTGF concentrations in mesangial cells overexpressing different levels of GLUT1. Supernatants from duplicate culture cells transduced with the GLUT1 gene denoted MCGT1 or a transfection control LaZ gene (MCLaZ).

Figure 10 depicts the effect of cyclic stretching on mesangial cell expression of CTGF transcripts. At the indicated periods, RNA was extracted and probed for CTGF message. Each lane represents the results of the samples pooled from 24 different culture wells.

Figure 11 depicts blockade of stimulated collagen type I production by and anti-CTGF antibody.

Figure 12 depicts blockade of stimulated mesangial cell proliferation by anti-CTGF antibody.

Figure 13A and Figure 13B depict glomerular disease associated with diabetes in db/db mice. Figure 13A shows renal cortical section from control *db/m* mice at 5 months of age. Figure 13B shows renal cortical section from diabetic *db/db* mice at 5 months of age. The

5 sections from Figure 13A and Figure 13B were stained with PAS for light microscopy examination, and are examples of glomeruli demonstrating the most severe mesangial expansion observed in the diabetic group.

Figure 14A, Figure 14B, and Figure 14C depict induction of CTGF and fibronectin transcripts in whole kidney of diabetic *db/db* mice at 5 months of age. Figures 14A and 14B show total RNA extracted from whole kidneys and probed by northern analysis for CTGF mRNA and fibronectin mRNA, respectively. The letter "C" represents nondiabetic mice, while the letter "D" represents diabetic mice. Figure 4C show a quantification by densometric analysis of the results of the Northern analyses

Figure 15A and Figure 15B depict competitive RT-PCR for GAPDH and CTGF mRNA in a single sample from diabetic mouse glomeruli. Ethidium bromide-stained gel after PCR amplification. The lanes of Figures 11A and 11B contain a constant amount of test cDNA and 2-fold decreasing concentrations of a known amount of the specific mimic. Figure 15A shows competitive reverse transcriptase PCR (RT-PCR) for GAPDH. Figure 15B shows competitive RT-PCR for CTGF.

Figure 16 depicts the effects of diabetes on the glomerular expression of CTGF and fibronectin transcript levels in *db/db* mice, detected by competitive RT-PCR.

Figure 17 depicts CTGF and its recovery in normal urine. Four aliquots were spiked with a different amount of CTGF, and a fifth served as a control. Immunoblotting was performed using a CTGF specific antibody.

Figure 18 depicts the analysis of CTGF protein in urine of diseased patients or healthy volunteers. Urine samples from 8 patients with kidney disease or 3 normal volunteers were assayed for CTGF by immunoblotting.

## V. DETAILED DESCRIPTION OF THE INVENTION

It is understood that the present invention is not limited to the particular methodologies, protocols, cell lines, and reagents, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular

embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, a reference to “an antibody” is a reference to one or more antibodies and any equivalents thereof known to those skilled in the art.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Preferred methods, devices, and materials are described, although any similar or equivalent methods can be used in the practice or testing of the present invention. All patents, publications, and other references cited herein are incorporated by reference herein in their entirety.

#### DEFINITIONS

As used herein, the term “extracellular matrix” refers broadly to non-cellular matrix, typically composed of proteins, glycoproteins, complex carbohydrates, and other macromolecules. Extracellular matrix components include, for example, collagen types I and IV, fibronectin, laminin, and thrombospondin.

The term “fibrosis” refers to abnormal processing of fibrous tissue, or fibroid or fibrous degeneration. Fibrosis can result from various injuries or diseases, and can often result from chronic transplant rejection relating to the transplantation of various organs. Fibrosis typically involves the abnormal production, accumulation, or deposition of extracellular matrix components, including overproduction and increased deposition of, for example, collagen and fibronectin.

As used herein, the terms “kidney fibrosis” or “renal fibrosis” or “fibrosis of the kidney” refer to diseases or disorders associated with the overproduction or abnormal deposition of extracellular matrix components, particularly collagen, leading to the degradation or impairment of kidney function. The terms “disorders” and “diseases” are used inclusively and refer to any condition deviating from normal. “Diseases” and “disorders” include, but are not limited to, allograft and transplant rejection, acute and chronic, and any transplant nephropathy; acute and

5 chronic kidney failure; autoimmune nephropathy; diabetic nephropathy; glomerulonephritis, glomerulosclerosis, and other forms of glomerular abnormality or injury; hypertension; hypertrophy; interstitial disease; nephritis; sclerosis, an induration or hardening of tissues and/or vessels resulting from causes that include, for example, inflammation due to disease or injury; renal-associated proliferative disorders; and other primary or secondary nephrogenic conditions.

10 Fibrosis associated with dialysis following kidney failure and catheter placement, e.g., peritoneal and vascular access fibrosis, is also included.

It is understood that, while kidney fibrosis is the model for discussion of the present invention, the mechanism of fibrosis is universal. Therefore, the presently described methods, kits, and other aspects of the present invention could also be directed to the diagnosis,

15 prevention, and treatment of other forms of fibrosis and diseases and disorders associated with fibrosis and proliferation, including, but not limited to: cardiac fibrosis, pulmonary fibrosis, diabetic retinopathy, skin fibrosis, scleroderma, atherosclerosis, arteriosclerosis, hypertrophic scarring, keloid formation, arthritis, liver fibrosis, inflammation, tumor growth metastasis, other conditions related to cell proliferation and migration, including those associated with vascular endothelial cells, for example, angiogenesis and neovascularization, etc.

20

The term “sample” is used herein in its broadest sense. Samples may be derived from any source, for example, from bodily fluids, secretions, or tissues including, but not limited to, saliva, blood, urine, and organ tissue (e.g., biopsied tissue); from chromosomes, organelles, or other membranes isolated from a cell; from genomic DNA, cDNA, RNA, mRNA, etc.; and from

25 cleared cells or tissues, or blots or imprints from such cells or tissues. A sample can be in solution or can be, for example, fixed or bound to a substrate. A sample can refer to any material suitable for testing for the presence of CTGF or suitable for screening for molecules that bind to CTGF or fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

30 An “antisense sequence” is any sequence capable of specifically hybridizing to a target sequence. The antisense sequence can be DNA, RNA, or any nucleic acid mimic or analog. The

5 term “antisense technology” refers to any technology which relies on the specific hybridization of an antisense sequence to a target sequence.

The terms “modulation” and “regulation” as used with respect to CTGF expression or activity refer to any direction of or effect on CTGF expression or activity as compared to normal or to unaltered CTGF expression or activity.

10

#### A. CTGF and its Role in Fibrosis and Renal Disorders

The present invention is based on the discovery that CTGF is an important mediator of extracellular accumulation in fibrotic conditions, and, in particular, in fibrotic conditions associated with renal disorders, such as diabetes and glomerular hypertension. More specifically, 15 the present invention is based on the discovery that the production of CTGF by glomerular cells (in particular, mesangial cells) is a potentially important factor in the pathogenesis of diseases and disorders of the kidney. It was discovered that increased levels of CTGF induced increased production and deposition of ECM in mesangial cells. It was further found in an analysis of urine samples that healthy subjects had no or very minimal levels of CTGF in their urine, while 20 the urine of diabetic patients or patients with other renal disorders showed increased levels of CTGF.

To demonstrate that CTGF is a critical determinant of extracellular matrix deposition in the kidney, CTGF expression in mesangial cells, glomeruli, and whole kidney was examined under diabetic and non-diabetic conditions. Mesangial cells cultured in media containing normal 25 levels of glucose expressed low levels of CTGF mRNA and secreted barely detectable amounts of the full length CTGF protein. However, in a hyperglycemic environment in which mesangial cells were exposed to elevated glucose levels, upregulation of CTGF expression and increased protein production were detected. Moreover, mechanical strain of mesangial cells, exhibitive of, for example, glomerulosclerosis, glomerular hypertension, and glomerular hypertrophy, 30 demonstrated an upregulated expression and protein production of CTGF in mesangial cells. Thus, the present invention demonstrates that exposure to conditions such as increased glucose concentrations, mechanical force, or TGF- $\beta$  led to upregulated expression and protein production

5 of CTGF, establishing a nexus between the presence of CTGF and renal disorders, in particular,  
diabetes and hypertension. Even in the absence of hypertension, conditions that produce, for  
example, glomerular hypertrophy, often a result of renal injury, can result in induce increased  
capillary vessel diameter. According to Laplace's law, vessel wall tension is correspondingly  
increased, and increased mesangial cell stretching forces are likely produced. See Cortes et al.,  
10 1997, *supra*.

### **B. Methods For Modulating And Inhibiting Activity Of CTGF**

Connective Tissue Growth Factor (CTGF) is a critical determinant of extracellular matrix  
deposition in kidney fibrotic conditions. The present invention provides methods for the  
15 diagnosis, prevention, and treatment of complications associated with kidney fibrosis, preferably,  
by regulating, modulating, and/or inhibiting the expression or activity of CTGF. More  
specifically, methods of the present invention provide for the administration of a therapeutically  
effective amount of an agent that regulates, modulates, and/or inhibits the extracellular matrix  
producing activity of CTGF.

20 **Antibodies.** In one embodiment of the present invention, methods for diagnosis,  
prevention, and treatment of renal disorders and diseases involve the administration of a  
therapeutically effective amount of an antibody which specifically reacts with a CTGF  
polypeptide or fragments thereof.

CTGF antibodies may be generated using methods well known in the art. Such antibodies  
25 may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain antibodies, as  
well as Fab fragments, including F(ab')<sub>2</sub> and F<sub>v</sub> fragments. Fragments can be produced, for  
example, by a Fab expression library. Neutralizing antibodies, i.e., those which inhibit dimer  
formation, are especially preferred for therapeutic use.

A target polypeptide, such as CTGF or an agent that modulates the activity and or  
30 expression of CTGF, can be evaluated to determine regions of high immunogenicity. Methods  
of analysis and epitope selection are well-known in the art. See, e.g., Ausubel, *et al.*, eds., 1988,  
*Current Protocols in Molecular Biology*. Analysis and selection can also be accomplished, for

5 example, by various software packages, such as LASERGENE NAVIGATOR software.  
(DNASTAR; Madison WI.) The peptides or fragments used to induce antibodies should be  
antigenic, but are not necessarily biologically active. Preferably, an antigenic fragment or  
peptide is at least 5 amino acids in length, more preferably, at least 10 amino acids in length, and  
most preferably, at least 15 amino acids in length. It is preferable that the antibody-inducing  
10 fragment or peptide is identical to at least a portion of the amino acid sequence of the target  
polypeptide, e.g., CTGF. A peptide or fragment that mimics at least a portion of the sequence of  
the naturally occurring target polypeptide can also be fused with another protein, e.g., keyhole  
limpet hemocyanin (KLH), and antibodies can be produced against the chimeric molecule.

Methods for the production of antibodies are well-known in the art. For example, various  
15 hosts, including goats, rabbits, rats, mice, humans, and others, may be immunized by injection  
with the target polypeptide or any immunogenic fragment or peptide thereof. Depending on the  
host species, various adjuvants may be used to increase immunological response. Such adjuvants  
include, but are not limited to, Freund's adjuvant, mineral gels such as aluminum hydroxide, and  
surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil  
20 emulsions, KLH, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-  
Guerin) and Corynebacterium parvum are especially preferable.

Monoclonal and polyclonal antibodies may be prepared using any technique which  
provides for the production of antibody molecules by continuous cell lines in culture.  
Techniques for *in vivo* and *in vitro* production are well-known in the art. See, e.g., Pound, J.D.,  
25 1998, Immunochemical Protocols, Humana Press, Totowa NJ; Harlow, E. and D. Lane, 1988,  
Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York. The production  
of chimeric antibodies is also well-known, as is the production of single-chain antibodies. See,  
e.g., Morrison, S. L. *et al.*, 1984, *Proc. Natl. Acad. Sci.* 81:6851-6855; Neuberger, M. S. *et al.*,  
1984, *Nature* 312:604-608; Takeda, S. *et al.*, 1985 *Nature* 314:452-454. Antibodies with related  
30 specificity, but of distinct idiotypic composition, may be generated, for example, by chain  
shuffling from random combinatorial immunoglobulin libraries. See, e.g., Burton D. R., 1991,  
*Proc. Natl. Acad. Sci.* 88:11120-11123.



Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents. *See, e.g.,* Orlandi, R. *et al.*, 1989, *Proc. Natl. Acad. Sci.* 86:3833-3837; Winter, G. and C. Milstein, 1991, *Nature* 349:293-299). Antibody fragments which contain specific binding sites for the target polypeptide may also be generated. Such antibody fragments include, but are not limited to, F(ab')<sub>2</sub> fragments, which can be produced by pepsin digestion of the antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. *See, e.g.,* Huse, W. D., *et al.*, 1989 *Science* 254:1275-1281.

Antibodies can be tested for anti-target polypeptide activity using a variety of methods well-known in the art. Various techniques may be used for screening to identify antibodies having the desired specificity, including various immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), including direct and ligand-capture ELISAs, radioimmunoassays (RIAs), immunoblotting, and fluorescent activated cell sorting (FACS). Numerous protocols for competitive binding or immunoradiometric assays, using either polyclonal or monoclonal antibodies with established specificities, are well known in the art. *See, e.g.,* Harlow and Lane. Such immunoassays typically involve the measurement of complex formation between the target polypeptide and a specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the target polypeptide is preferred, but other assays, such as a competitive binding assay, may also be employed. *See, e.g.,* Maddox, D. E., *et al.*, 1983, *J Exp Med* 158:1211.

Antibodies as described above could also be used to identify CTGF or fragments thereof in tissue, e.g., from a kidney biopsy. The amount of CTGF present could be determined, for example, by quantitative image analysis. CTGF mRNA levels could also be determined, such as by reverse transcriptase polymerase chain reaction (PCR) using portions of the biopsied tissue, e.g., glomeruli. In particular, in this method, mRNA from a tissue sample, in total, or that specific for CTGF or fragments thereof, could be transcribed to DNA and then amplified through

5 PCR using CTGF-specific primer sequences. Quantitation of mRNA for CTGF or fragments thereof could be determined, for example, by a competition reaction using equal volumes of the patient sample run against a series of decreasing known concentrations, e.g., of a mimic or mutant cDNA fragment.

10 The present invention contemplates the use of antibodies specifically reactive with a CTGF polypeptide or fragments thereof which neutralize the biological activity of CTGF. The antibody administered in the method can be the intact antibody or antigen binding fragments thereof, such as Fab, F(ab')<sub>2</sub>, and F<sub>v</sub> fragments, which are capable of binding the epitopic determinant. The antibodies used in the method can be polyclonal or, more preferably, monoclonal antibodies. Monoclonal antibodies with different epitopic specificities are made  
15 from antigen containing fragments of the protein by methods well known in the art. *See, e.g., Kohler et al., Nature 256:494; Ausubel, et al., supra.*

20 In the present invention, therapeutic applications include those using "human" or "humanized" antibodies directed to CTGF or fragments thereof. Humanized antibodies are antibodies, or antibody fragments, that have the same binding specificity as a parent antibody, (i.e., typically of mouse origin) and increased human characteristics. Humanized antibodies may be obtained, for example, by chain shuffling or by using phage display technology. For example, a polypeptide comprising a heavy or light chain variable domain of a non-human antibody specific for a CTGF is combined with a repertoire of human complementary (light or heavy) chain variable domains. Hybrid pairings specific for the antigen of interest are selected. Human  
25 chains from the selected pairings may then be combined with a repertoire of human complementary variable domains (heavy or light) and humanized antibody polypeptide dimers can be selected for binding specificity for an antigen. Techniques described for generation of humanized antibodies that can be used in the method of the present invention are disclosed in, for example, U.S. Patent Nos. 5,565,332; 5,585,089; 5,694,761; and 5,693,762. Furthermore,  
30 techniques described for the production of human antibodies in transgenic mice are described in, for example, U.S. Patent Nos. 5,545,806 and 5,569,825.

5 In another embodiment of the present invention, a method involves the administration of  
a therapeutically effective amount of an antibody reactive to a CTGF responsive receptor, and,  
more specifically, an antibody which blocks the binding of CTGF to its cellular receptors. The  
method of the present invention provides that the antibody reactive with CTGF modulates and/or  
inhibits the biological activity of CTGF through the manipulation and control of the interaction  
10 between CTGF and its receptor by inactivation of the receptor independently of CTGF.

**Antisense Oligonucleotides.** The present invention provides for a therapeutic approach  
which directly interferes with CTGF expression. Specifically, a therapeutic approach which  
directly interrupts the translation of CTGF mRNA into protein could be used to bind to CTGF  
mRNA or to otherwise interfere with CTGF expression. Antisense technology relies on the  
15 modulation of expression of a target protein through the specific binding of an antisense  
sequence to a target sequence encoding the target protein or directing its expression. *See, e.g.,*  
Agrawal, S., ed., 1996, *Antisense Therapeutics*, Humana Press Inc., Totawa NJ; Alama, A. *et*  
*al.*, 1997, *Pharmacol. Res.* 36(3):171-178; Crooke, S.T., 1997, *Adv. Pharmacol.* 40:1-49; and  
Lavrosky, Y. *et al.*, 1997, *Biochem. Mol. Med.* 62(1):11-22. Antisense sequences are nucleic acid  
20 sequences capable of specifically hybridizing to at least a portion of a target sequence. Antisense  
sequences can bind to cellular mRNA or genomic DNA, blocking translation or transcription and  
thus interfering with expression of a targeted protein product. Antisense sequences can be any  
nucleic acid material, including DNA, RNA, or any nucleic acid mimics or analogs. *See, e.g.,*  
Rossi, J.J. *et al.*, 1991 *Antisense Res. Dev.* 1(3):285-288; Pardridge, W.M. *et al.*, 1995, *Proc.*  
25 *Nat. Acad. Sci.* 92(12):5592-5596; Nielsen, P.E. and G. Haaima, 1997, *Chem. Soc. Rev.* 96:73-  
78; and Lee, R. *et al.*, 1998, *Biochemistry* 37(3):900-1010.). Delivery of antisense sequences can  
be accomplished in a variety of ways, such as through intracellular delivery using an expression  
vector. See discussion, *infra*. Site-specific delivery of exogenous genes is also contemplated,  
such as techniques in which cells are first transfected in culture and stable transfectants are  
30 subsequently delivered to the target site. *See, e.g.,* Kitamura, M. *et al.*, 1994, *Kidney Int.* 43:S55-  
S58.

5 Antisense oligonucleotides of about 15 to 25 nucleic acid bases are typically preferred as such are easily synthesized and are capable of producing the desired inhibitory effect. Molecular analogs of antisense oligonucleotide may also be used for this purpose and can have added advantages such as stability, distribution, or limited toxicity advantageous in a pharmaceutical product. In addition, chemically reactive groups, such as iron-linked ethylenediamine-tetraacetic  
10 acid (EDTA-Fe), can be attached to antisense oligonucleotides, causing cleavage of the RNA at the site of hybridization. These and other uses of antisense methods to inhibit the *in vitro* translation of genes are well known in the art. See, e.g., Marcus-Sakura, 1988, *Anal. Biochem* 172:289.

15 Delivery of antisense therapies and the like can be achieved intracellularly through using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system which, upon transcription, produces a sequence complementary to at least a portion of the cellular sequence encoding the target protein. See, e.g., Slater, J.E. et al., 1998, *J. Allergy Clin. Immunol.* 102(3):469-475. Delivery of antisense sequences can also be achieved through various viral vectors, including retrovirus and adeno-associated virus vectors. See, e.g., Miller, A.D., 1990, *Blood* 76:271; and Uckert, W. and W. Walther, 1994, *Pharmacol. Ther.* 63(3):323-347. Vectors which can be utilized for antisense gene therapy as taught herein include, but are not limited to, adenoviruses, herpes viruses, vaccinia, or, preferably, RNA viruses such as retroviruses.

20 Retroviral vectors are preferably derivatives of murine or avian retrovirus. Retroviral vectors can be made target-specific by inserting, for example, a polynucleotide encoding a protein or proteins such that the desired ligand is expressed on the surface of the viral vector. Such ligand may be a glycolipid carbohydrate or protein in nature. Preferred targeting may also be accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of  
25 the retroviral vector containing the antisense polynucleotide.

30 Recombinant retroviruses are typically replication defective, and can require assistance in order to produce infectious vector particles. This assistance can be provided by, for example,

5 using helper cell lines that contain plasmids encoding all-of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal may be used. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Other gene delivery mechanisms that can be used for delivery of antisense sequences to target cells include colloidal dispersion and liposome-derived systems, artificial viral envelopes, and other systems available to one of skill in the art. See, e.g., Rossi, J.J., 1995, Br. Med. Bull. 51(1):217-225; Morris, M.C. *et al.*, 1997, *Nucl. Acids Res.* 25(14):2730-2736; and Boado, R.J. *et al.*, 1998, *J. Pharm. Sci.* 87(11):1308-1315. For example, delivery systems can make use of macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

In one embodiment, a method of the present invention administers a therapeutically effective amount of an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of an mRNA molecule which encodes CTGF, so as to prevent translation of CTGF mRNA.

In another embodiment of the present invention, a method is provided in which a therapeutically effective amount of an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of CTGF mRNA so as to prevent translation of the mRNA.

**Small Molecule Inhibitors.** The present invention further provides a method in which small molecules are used to inhibit the activity of CTGF by blocking the binding of responsive cytokines to the CTGF responsive receptor. For example, the present invention provides methods of treating and preventing kidney fibrosis utilizing small molecules that modulate, regulate and inhibit CTGF activity.

5 In order to identify small molecules and other agents useful in the present methods for treating or preventing a renal disorder by modulating the activity and expression of CTGF, CTGF and biologically active fragments thereof can be used for screening therapeutic compounds in any of a variety of screening techniques. Fragments employed in such screening tests may be free in solution, affixed to a solid support, borne on a cell surface, or located  
10 intracellularly. The blocking or reduction of biological activity or the formation of binding complexes between CTGF and the agent being tested can be measured by methods available in the art.

Other techniques for drug screening which provide for a high throughput screening of compounds having suitable binding affinity to CTGF, or to another target polypeptide useful in  
15 modulating, regulating, or inhibiting the expression and/or activity of CTGF, are known in the art. For example, microarrays carrying test compounds can be prepared, used, and analyzed using methods available in the art. *See, e.g.,* Shalon, D. *et al.*, 1995, PCT Application No. WO95/35505, Baldeschweiler *et al.*, 1995, PCT Application No. WO95/251116; Brennan, T.M. *et al.*, 1995, U.S. Patent No. 5,474,796; Heller, M.J. *et al.*, 1997, U.S. Patent No. 5, 605,662.

20 Identifying small molecules that modulate CTGF activity can also be conducted by various other screening techniques, which can also serve to identify antibodies and other compounds that interact with CTGF and can be used as drugs and therapeutics in the present methods. *See, e.g.,* Enna, S.J. *et al.*, eds., 1998, Current Protocols in Pharmacology, John Wiley and Sons. Assays will typically provide for detectable signals associated with the binding of the  
25 compound to a protein or cellular target. Binding can be detected by, for example, fluorophores, enzyme conjugates, and other detectable labels well-known in the art. *See, e.g.,* Enna *et al.*. The results may be qualitative or quantitative.

For screening the compounds for specific binding, various immunoassays may be employed for detecting, for example, human or primate antibodies bound to the cells. Thus, one  
30 may use labeled anti-hlg, *e.g.,* anti-hlgM, hlgG or combinations thereof to detect specifically bound human antibody of the galactosyl epitope. Various labels can be used such as radioisotopes, enzymes, fluorescers, chemiluminescers, particles, etc. There are numerous

5 commercially available kits providing labeled anti-hIg, which may be employed in accordance with the manufacturer's protocol.

For screening the compounds for cytotoxic effects, a wide variety of protocols may be employed to ensure that one has the desired activity. One will normally use cells, which may be naturally occurring or modified, cell lines, or the like. The cells may be prokaryotic or  
10 eukaryotic. For example, if one is interested in a pathogen, where it does not matter to which epitope the compound conjugate binds, one can combine the pathogenic cells with each of the compounds in the presence of an antibody dependent cytotoxic system to determine the cytotoxic effect. One may perform this assay either prior to or subsequent to determining the effect of the various candidate compounds on cells of the host to whom the compound would be administered.  
15 In this way, one would obtain a differential analysis between the affinity for the pathogenic target and the affinity for host cells which might be encountered, based on the mode of administration.

In some situations, one would be interested in a particular cellular status, such as an activated state, as may be present with T cells in autoimmune diseases, transplantation, and the like. In this situation one would first screen the compounds to determine those which bind to the quiescent cell, and as to those compounds which are not binding to the quiescent cells, and  
20 screen the remaining candidate compounds for cytotoxicity to the activated cells. One may then screen for other cells present in the host which might be encountered by the compounds to determine their cytotoxic effect. Alternatively, one might employ cancer cells and normal cells to determine whether any of the compounds have higher affinity for the cancer cells, as  
25 compared to the normal cells. Again, one could screen the library of compounds for binding to normal cells and determine the effect. Those compounds which are not cytotoxic to normal cells could then be screened for their cytotoxic effect to cancer cells. Even where some cytotoxicity exists for normal cells, in the case of cancer cells, where there is a sufficient differentiation in cytotoxic activity, one might be willing to tolerate the lower cytotoxicity for normal cells, where  
30 the compound is otherwise shown to be effective with cancer cells.

Instead of using cells which are obtained naturally, one may use cells which have been modified by recombinant techniques. Thus, one may employ cells which can be grown in

5 culture, which can be modified by upregulating or downregulating a particular gene. In this way,  
one would have cells that differ as to a single protein on the surface. One could then  
differentially assay the library as to the effect of members of the library on cells for which the  
particular protein is present or absent. In this way, one could determine whether the compound  
has specific affinity for a particular surface membrane protein as distinct from any of the proteins  
10 present on the surface membrane.

One may differentiate between cells by using antibodies binding to a particular surface  
membrane protein, where the antibodies do not initiate the complement dependent cytotoxic  
effect, for example, using different species, isotypes, or combinations thereof. By adding the  
antibodies, blocking antisera or monoclonal antibodies, to one portion of the cells, those cells  
15 will not have the target protein available for binding to the library member. In this way one  
creates comparative cells which differ in their response based on the unavailability in one group  
of a single protein. While antibodies will usually be the most convenient reagent to use, other  
specific binding entities may be employed which provide the same function.

For use in the assay to determine binding, one may use an antibody-dependent cytotoxic  
20 system. One could use synthetic mixtures of the ingredients, where only those components  
necessary for the cytotoxic effect are present. This may be desirable where components of blood  
or plasma may adversely affect the results of the assay.

Also, while a cellular lawn is an extremely convenient way to screen large numbers of  
candidates, other techniques may also find use. These techniques include the use of multiwell  
25 plates, and the various devices used for the preparation of the combinatorial library, such as pins,  
tea bags, etc. One may grow the cells separately in relation to the nature of the various devices,  
where the device may then be contacted with the cells or have the cells grown on the device. The  
device may be immersed in an appropriate culture, seeded with the cells, or otherwise provided  
for contact between the cells and the candidate compound. After adding the cytotoxic agent, one  
30 may then analyze for lysis in a variety of ways. For example, FACS may be used for  
distinguishing between live and dead cells, sup 51 Cr release may be employed, or detection of  
an intracellular compound in the supernatant, may serve to detect active compounds.



5 In addition, one may wish to know whether the compound has agonist or antagonist activity. The subject assay techniques provide for a rapid way for determining those compounds present in the library which bind to the target protein. Once, one has substantially narrowed the number of candidate compounds, one can use more sophisticated assays for detecting the activity of the compound itself. In this way, one can perform a rapid screen to determine binding affinity and specificity, followed by a more intensive screen to determine activity. Various techniques exist for determining activity, where the cells may be modified, so that a marker gene will be activated which will provide for a detectable signal. Conveniently, the signal may be associated with production of a dye, the production of a surface membrane protein which can be detected with labeled antibodies, or the secretion of a protein which can be detected in the supernatant by any of a variety of techniques. For example, the gene that is expressed may be luciferase modified to have a leader sequence so as to be secreted, whereby the supernatant can then be screened for light generation formation by using an appropriate substrate.

Various protocols may be employed for screening the library. To some degree, this will depend upon the nature of the preparation of the compounds. For example, the compounds may be bound to individual particles, pins, membranes, or the like, where each of the compounds is segregatable. In addition, the amount of compound available will vary, depending upon the method employed for creating the library. Furthermore, depending upon the nature of the attachment of the compound to the support, one may be able to release aliquots of a compound, so as to carry out a series of assays. In addition, the manner in which the compounds are assayed will be affected by the ability to identify the compound which is shown to have activity.

Where the compounds are individually on a surface in a grid, so that at each site of the grid one knows what the composition is, one can provide a cellular lawn which is similarly organized as a grid and may be placed in registry with the compounds bound to the solid surface. Once the lawn and solid substrate are in registry, one may release the compounds from the surface in accordance with the manner in which the compounds are attached. After sufficient time for the compounds to bind to the proteins on the cellular surface, one may wash the cellular lawn to remove non-specifically bound compounds. One or more washings may be involved,

5 where the washings may provide for varying degrees of stringency, depending upon the desired degree of affinity. After the washings have been completed, mammalian blood or plasma may then be added and incubated for sufficient time for cytotoxicity. The plasma or blood may then be removed and plaques observed, where the nature of the compound can be determined by virtue of the position in the grid. The plasma or blood can be free of any components that would  
10 naturally kill the cells of the lawn.

Since the preparative process may be repeated, one could prepare a plurality of solid substrates, where the same compounds are prepared at the comparable sites, so that the screening could be repeated with the same or different cells to determine the activity of the individual compounds. In some instances, the identity of the compound can be determined by a nucleic acid tag, using the polymerase chain reaction for amplification of the tag. *See, e.g.*, PCT  
15 Application No. WO93/20242. In this instance, the compounds that are active may be determined by taking the lysate and introducing the lysate into a polymerase chain reaction medium comprising primers specific for the nucleic acid tag. Upon expansion, one can sequence the nucleic acid tag or determine its sequence by other means, which will direct the selection of  
20 the procedure is used to prepare the compound.

Alternatively, one may have tagged particles where the tags are releasable from the particle and provide a binary code that describes the synthetic procedure for the compounds bound to the particle. *See, e.g.*, Ohlmeyer, et al., 1993, *PNAS* 90:10922. These tags can conveniently be a homologous series of alkylene compounds, which can be detected by gas  
25 chromatography-electron capture. Depending upon the nature of the linking group, one may provide for partial release from the particles, so that the particles may be used 2 or 3 times before identifying the particular compound.

While for the most part libraries have been discussed, any large group of compounds can be screened analogously, so long as the CTGF epitope can be joined to each of the compounds.  
30 Thus, compounds from different sources, both natural and synthetic, including macrolides, oligopeptides, ribonucleic acids, dendrimers, etc., may also be screened in an analogous manner.

5           Formation of a plaque in the assay demonstrates that binding of the member of the library to the cell, usually a surface protein, does not interfere with the CTGF epitope binding to an antibody, that the immune complex is sufficiently stable to initiate the complement cascade, and that the member has a high affinity for the target.

10           The subject methodology can be used in any situation where one has a cellular target to be killed, particularly those cellular targets having low or no CTGF epitope. Thus, the cellular target may be a prokaryote, which is pathogenic. Various organisms include, for example, microbacterium, *Yersinia*, *Pseudomonas*, *Bordetella pertussis*, *Treponema pallidum*, *Neisseria gonorrhoea*, *Streptococcus*, *Hemophilus influenza*, etc. Other pathogens include eukaryotes, particularly fungi, such as *Candida*, *Histoplasma*, etc., and protozoa, e.g., *Giardia*. In addition, 15 viruses which provide for surface membrane proteins in infected cells, can also be the target of the subject compounds, where the cells that are screened have been vitally infected.

20           Host cells may also serve as targets, where the cells are either abnormal or act in an adverse way to the host or treatments of the host. For example, cancerous tissues which can be distinguished from normal tissue can serve as a target for the subject compounds. T or B cells associated with autoimmune diseases or associated with GVHD or transplant rejection may also serve as targets. Aberrant cells, regardless of their nature, so long as they can be distinguished from normal cells, may also serve as targets. Thus, psoriatic lesions, lymphoma cells, bacterial, fungal, parasitic, virus infected cells, may be targets of the subject products. Also, where one wishes to ablate a portion of cells, without removal of all of the cells, such as cells expressing a 25 differentiation marker such as T cell subsets, activated platelets, endothelial cells, hormone or cytokine receptor expressing cells, the subject compounds may find application.

          Other screening methods for obtaining small molecules that modulate the activity of CTGF can be found, for example, PCT Application No. WO 9813353.

30           **Compounds/Molecules.** The present invention provides methods for treating and preventing disorders associated with kidney fibrosis by modulating, regulating, or inhibiting the activity of CTGF. These methods can comprise the administration of a therapeutically effective amount of a compound that blocks the binding interactions or blocks enzymes involved in the

5 signal transduction pathway of CTGF. More specifically, the present invention provides a method for inhibiting the activity of CTGF by administering compounds that block the induction of CTGF.

Compounds that modulate CTGF gene expression and/or CTGF activity in the method of the invention include agents which cause an elevation in cyclic nucleotides in the cell. Other  
10 compounds that may block the induction of CTGF according to the methods of the present invention, may be identified using the screening methods described above.

In yet a further embodiment of the present invention, the method provides for the administration of molecules that interrupt the post-translational modification of full length CTGF or block the activation of an inactive precursor of CTGF. As discussed herein, exposure of  
15 mesangial cells to TGF- $\beta$  resulted in the marked appearance of additional bands at 28-30kDa which correspond in size to the carboxy- and amino-terminal halves of the full length CTGF molecule. As disclosed above, TGF- $\beta$  treatment may result in the production of proteases or other factors capable of cleaving the full-length molecule. Molecules that inhibit CTGF activity may be identified using the screening methods provided herein.

20 The methods of the present invention may further be used to prevent or treat fibrosis in the kidney associated with allograft rejection comprising administering a therapeutically effective amount of any one of the agents described above.

The invention further provides a method for treating or preventing diabetes by administering an effective amount of insulin and an effective amount of an agent that regulates,  
25 modulates, or inhibits CTGF activity as described above.

### C. Pharmaceutical Formulations And Routes Of Administration

**Routes of Administration.** The antibodies, small molecules, and other compounds described herein can be administered to a human patient *per se*, or in pharmaceutical compositions comprising, where appropriate, suitable carriers or excipients. The present  
30 invention contemplates methods of treatment in which agents that modulate or regulate the expression or activity of CTGF or fragments thereof are administered to a patient in need, in amounts suitable to treat or prevent the overproduction of ECM associated with CTGF. The

5 present methods of treatment and prevention can comprise administration of an effective amount of the agent to a subject which is preferably a mammalian subject, and most preferably a human subject. In a preferred embodiment, the subject mammal and the agent administered are of homologous origin. Most preferably, the subject and the agent administered are human in origin.

10 An effective amount can readily be determined by routine experiment, as can the most effective and convenient route of administration and the most appropriate formulation. Various formulations and drug delivery systems are available in the art. *See, e.g.,* Gennaro, A.R., *ed.*, 1990, *Remington's Pharmaceutical Sciences*, 18<sup>th</sup> ed., Mack Publishing Co., Easton PA. Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration and parenteral delivery, including intramuscular, subcutaneous, 15 intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. The composition may be administered in a local rather than a systemic manner. For example, a composition comprising an agent which modulates, regulates, or inhibits the activity of CTGF can be delivered via injection or in a targeted drug delivery system into an area in which there is excess circulating CTGF or ECM overproduction or into an area in which inhibition of CTGF activity is desired, often in a depot or 20 sustained release formulation.

25 The pharmaceutical compositions of the present invention may be manufactured by any of the methods well-known in the art, such as by conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. As noted above, the compositions of the present invention can include one or more physiologically acceptable carriers such as excipients and auxiliaries which facilitate processing of active molecules into preparations for pharmaceutical use. Proper formulation is dependent upon the route of administration chosen.

30 For injection, for example, the composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the

5 art. For oral administration, the compounds can be formulated readily by combining the active  
compounds with pharmaceutically acceptable carriers well known in the art. Such carriers  
enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules,  
liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject. The  
compounds may also be formulated in rectal compositions such as suppositories or retention  
10 enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical preparations for oral use can be obtained as solid excipients, optionally  
grinding a resulting mixture, and processing the mixture of granules, after adding suitable  
auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular,  
fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations  
15 such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum  
tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose,  
and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the  
cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar  
20 solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone,  
carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable  
organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or  
dragee coatings for identification or to characterize different combinations of active compound  
doses.

25 Pharmaceutical preparations for oral administration include push-fit capsules made of  
gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or  
sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as  
lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and,  
optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in  
30 suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition,  
stabilizers may be added. All formulations for oral administration should be in dosages suitable  
for such administration.

5 For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or any other suitable gas. In the case of a pressurized aerosol, the appropriate dosage unit may be  
10 determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin, for use in an inhaler or insufflator may be formulated. These typically contain a powder mix of the compound and a suitable powder base such as lactose or starch.

Compositions formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion can be presented in unit dosage form, e.g., in ampoules or in  
15 multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Formulations for parenteral administration include aqueous solutions of agents that effect the activity of CTGF or fragments thereof, in water-soluble form.

20 Suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil and synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also  
25 contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compositions of the present invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example,  
30 subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as

an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical carriers for the hydrophobic molecules of the invention could include co-solvent systems comprising, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The co-solvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system is effective in dissolving hydrophobic compounds and produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied. For example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80, the fraction size of polyethylene glycol may be varied, other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone, and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic molecules may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using sustained-release systems, such as semi-permeable matrices of solid hydrophobic polymers containing the effective amount of the composition to be administered. Various sustained-release materials are established and available to those of skill in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

***Effective Dosage.*** For any composition used in the present methods of treatment, a therapeutically effective dose can be estimated initially using a variety of techniques well-known



in the art. For example, in a cell culture assay, a dose can be formulated in animal models to achieve a circulating concentration range that includes the  $IC_{50}$  as determined in cell culture. Where inhibition of CTGF activity is desired, for example, the concentration of the test compound which achieves a half-maximal inhibition of CTGF activity can be determined. Dosage ranges appropriate for human subjects can be determined using data obtained from cell culture assays and other animal studies.

A therapeutically effective dose refers to that amount of the molecule that results in amelioration of symptoms or a prolongation of survival in a subject. Toxicity and therapeutic efficacy of such molecules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the  $LD_{50}$  (the dose lethal to 50% of the population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, which can be expressed as the ratio  $LD_{50}/ED_{50}$ . Molecules which exhibit high therapeutic indices are preferred.

Dosages preferably fall within a range of circulating concentrations that includes the  $ED_{50}$  with little or no toxicity. Dosages may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage will be chosen in view of the specifics of a subject's condition.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to modulate or regulate CTGF activity as desired, i.e. minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from, for example, *in vitro* data, such as the concentration necessary to achieve 50-90% activity of CTGF to induce bone growth using the assays described herein.

Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Compositions should be administered using a regimen which maintains plasma levels above the MEC for about 10-90% of the duration of treatment, preferably about 30-90% of the duration of treatment, and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

5 The amount of composition administered will, of course, be dependent on a number of factors, including, but not limited to, the particular subject's weight, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician.

**Packaging.** The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack  
10 may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of disorders or diseases in  
15 which cartilage or bone induction, wound healing, neuroprotection or the like is desired.

**Receptor-Ligand Complexes.** As a consequence of the above described screening techniques, as well as other known screening techniques which may be applied in the context of the present invention, CTGF-ligand complexes can be formed. These complexes may include complexes wherein the ligand is a CTGF antagonist, a CTGF agonist, or any another compound capable of modulating the expression and activity of CTGF. Partial agonists or antagonists of the CTGF receptor may be useful for therapeutic or diagnostic purposes. CTGF-agent  
20 complexes may be useful as therapeutic entities in their own right or in methods of detecting and quantifying CTGF levels in a sample. The measurement and quantification of CTGF, for example, through the detection of CTGF-ligand complexes, can be accomplished by methods  
25 available in the art. *See, e.g., Enna et al.*

#### **D. Diagnostics**

The present invention is further directed to a method of detecting or diagnosing the presence of pathology of a tissue characterized by an excessive accumulation of the extracellular matrix components, in particular, those associated with renal disorders. One method involves the  
30 detection or diagnosis of diabetes, including diabetic nephropathy and diabetic glomerulosclerosis. In a preferred method, the detection or diagnosis is accomplished by measuring CTGF levels in a urine sample from a patient. In one embodiment, the method

5 includes determining the level of CTGF in a first urine sample and comparing this level to the  
level of CTGF present in a normal urine sample, i.e., a sample from a subject without a renal  
disorder. An elevated level of CTGF in the first sample is indicative of the pathological condition  
in question, for example, diabetes or hypertension. In particular, individuals without any renal  
disorders, normal levels of CTGF may be at or close to zero. In diabetic patients or in patients  
10 experiencing infection or other trauma, levels of CTGF may be significantly increased. Thus, the  
presence of kidney fibrosis could be identified by detecting increases in levels of CTGF in a  
sample. In a preferred method, the sample is a non-intrusive sample such as a urine sample.  
Assessment of CTGF levels in a urine sample can be accomplished, for example, by ELISA  
using a CTGF-specific antibody. Detection of CTGF levels could be indicative of the  
15 advancement or worsening of diabetic hypertension or other renal disorders prior to the onset of  
renal complications, providing for a method of early-stage detection and diagnosis. Furthermore,  
CTGF levels can serve as a predictor of, for example, which diabetic patients have a  
predisposition to develop kidney diseases and disorders.

20 More generally, detection of CTGF levels, including levels of unique forms or fragments  
of CTGF, may be obtained through immunoassay methods, for example, ELISAs, RIAs, or any  
other assays which utilize an antibody to detect the presence of a protein marker. The ELISA  
and RIA methods are preferred and may be used, for example, with the monoclonal antibodies of  
the present invention to detect levels of CTGF. In a preferred method of the invention, urine  
samples are obtained first from patients suspected or known to have a renal disease or disorder.  
25 Levels of CTGF in this first sample are measured, for example, through immunoassay, and are  
compared with the CTGF levels in a second sample, the second sample being obtained from a  
patient known to have a renal disorder or from a patient known not to have any renal disorder, to  
determine the presence or progression of a kidney disease. The same methods may be used to  
monitor the progression of a kidney disease.

30 More generally, antibodies specific for a target polypeptide, such as antibodies specific  
for CTGF, are useful in the present invention for diagnosis of renal disorders and diseases  
associated with aberrant expression of CTGF. Diagnostic assays for CTGF can include methods

utilizing the antibody and a label to detect CTGF in a sample from a patient suspected of having a renal disorder or disease. The sample could comprise, for example, body fluids, cells, tissues, or extracts of such tissues, including, for example, glomeruli microdissected from biopsy material. Protocols employed to screen for and identify antibodies having the desired specificity can also be used for the detection of CTGF or the target polypeptide in the sample.

Preferably, in the diagnostic methods of the present invention, normal or standard values for CTGF expression are established in order to provide a basis for the diagnosis of the existence of a renal disease or disorder or a predisposition to a renal disease or disorder. In one of the methods of the present invention, this is accomplished by combining body fluids or cell extracts taken from normal subjects with antibody to CTGF under conditions suitable for complex formation. Such conditions are well known in the art. The amount of standard complex formation may be quantified by comparing levels of antibody-target complex in the normal sample with a dilution series of positive controls, in which a known amount of antibody is combined with known concentrations of purified CTGF. Standard values obtained from normal samples may be compared, for example, in a specific embodiment, with values obtained from samples from subjects suspected of having a kidney disease or disorder, or having a predisposition to a kidney disease or disorder, associated with kidney fibrosis. Deviation between standard and subject values establishes the presence of or predisposition to the disease state. The diagnostic methods of the present invention may also be directed to the detection of a predisposition or susceptibility to a renal disorder. This can be accomplished, for example, by detecting a marker indicative of a predisposition or susceptibility to develop a particular disorder, for example, diabetes. The marker can comprise, for example, a genetic polymorphism.

Monoclonal antibodies can be detected by methods discussed, for example, *supra*. Monoclonal antibodies against CTGF can be conjugated to an appropriate enzyme such as horseradish peroxidase, protein ferritin, enzyme alkaline phosphatase,  $\beta$ -D-galactosidase etc. These enzyme-linked antibody preparations can be mixed with, for example, urine samples that contain unknown amounts of CTGF in an indirect ELISA. Direct or sandwich ELISAs could also be performed using the same antibodies.

5 RIA techniques may also be used to measure levels of CTGF in, for example, urine. For example, CTGF may be radioactively labeled and mixed with monoclonal antibodies specific for CTGF and a serum sample containing an unknown amount of unlabeled CTGF. Binding competition between the labeled and unlabeled CTGF with the monoclonal antibody occurs. By measuring the amount of radioactivity of the reaction mixture, the amount of CTGF present in  
10 the sample can be quantitatively determined. *See, e.g.*, US Patent Nos. 4,438,209 and 4,591,573. Non-competitive RIAs can also be performed.

Polynucleotide sequences encoding CTGF can be used for the diagnosis of conditions or diseases associated with increased levels of CTGF expression. For example, polynucleotide sequences encoding CTGF may be used in hybridization or PCR assays of fluids or tissues from  
15 biopsies to detect CTGF expression. The form of such qualitative or quantitative methods may include Southern or northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin, chip and ELISA technologies. All of these techniques are well known in the art and are the basis of many commercially available diagnostic kits.

The present invention additionally provides methods for evaluating the effectiveness of anti-fibrotic therapy, including the use of ACE inhibitors, by measuring the levels of CTGF in a  
20 sample from a subject undergoing a course of treatment for diseases and disorders associated with fibrosis. CTGF levels can be measured in samples, for example, urine sample, taken from the subject at various points before, during, and after a course of treatment. The efficacy of a treatment can be evaluated with reference to the variation in CTGF levels present in the samples  
25 taken at different stages of a course of treatment.

**Kits.** The present invention provides kits for detecting CTGF in samples, in particular, in fluid samples. In a preferred embodiment, the diagnostic kits of the present invention contains reagents for measuring levels of CTGF in urine samples. In a particular embodiment, this kit comprises a monoclonal antibody specific for CTGF bound to a support and a second  
30 monoclonal antibody specific for a different CTGF epitope and enzyme-labeled. The kit further comprises reagents for detecting the enzyme-labeled monoclonal antibody. The reagent kit employs immunological methods in measuring CTGF in the urine sample, thus allowing for the

5 detection and monitoring of kidney disorders and diseases. In particular embodiments, the kit allows for the detection and monitoring of fibrotic and sclerotic disorders resulting from, for example, diabetes and hypertension. In another embodiment, the kit comprises a radio-labeled or fluorescein labeled antibody in place of the enzyme-labeled antibody.

10 In one embodiment, the diagnostic kit of the present invention comprises elements useful in the detection of CTGF in tissue samples, using immunohistochemical techniques. The kit could be used in conjunction with, for example, a software program which allows for quantitative measurement of the levels of CTGF in the tissue sample by image analysis or other comparative techniques. *See, e.g., Riser et al., 1996, Am. J. Path., supra.* Another embodiment provides a diagnostic kit for detecting and measuring levels of CTGF mRNA in tissue samples. In one  
15 embodiment, the kit comprises reagents used to reverse transcribe CTGF mRNA to DNA. The kit can further comprise reagents necessary to amplify CTGF-specific DNA, including primers complementary to polynucleotides encoding CTGF or fragments thereof. The kit can also include a competitive mimic or mutant cDNA for use in quantifying the level of CTGF mRNA present in the sample.

20 In a preferred embodiment, the diagnostic kit of the present invention is packaged and labeled, for example, in box or container which includes the necessary elements of the kit, and includes directions and instructions on the use of the diagnostic kit.

25 The following examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become  
30 apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

## 5 VI. EXAMPLES

Unless otherwise stated, the following materials and methods were used in the examples of the present invention.

**Cells And Tissue Culture.** The mesangial cells were a cloned-line derived from outgrowths of Fischer rat glomeruli, and upon serial passage, these mesangial cells continue to express key markers (See, e.g., Riser *et al.*, 1998, *J. Am. Soc. Nephrol.* 9:827-836). The medium used was RPMI 1640 with penicillin and streptomycin and, unless otherwise noted, 5 mM glucose. The growth medium contained 20% Nu-serum (Collaborative Research, Bedford, MA). Unless otherwise noted, mesangial cells were cultured for approximately 4 days in growth medium, and when reaching confluency, were washed twice with serum-free medium and incubated for 24-48 hours under serum-deprived of 0.5% FCS (fetal calf serum) conditions. The cultures were then incubated for a designated period in fresh maintenance medium (0.5 % FCS), with or without experimental treatments. At the concentration of FCS used in these studies, no active TGF- $\beta$ 1, TGF- $\beta$ 2, or TGF- $\beta$ 3 was detectable in the fresh medium, as determined by a highly sensitive mink lung bioassay. (See, e.g., Riser *et al.*, 1996, *Am. J. Pathology, supra.*) The renal fibroblasts used in Northern analysis were mouse tubulointerstitial fibroblasts (TFB). (See, e.g., Alvarez, *et. al.*, 1998, *Kidney Int.* 41:14-23.)

**Animals And Specimen Collection.** Diabetic male *db/db* mice and their nondiabetic *db/m* littermates were obtained from Jackson Laboratories (Bar Harbor, ME). The *db/db* mouse carries a defective receptor gene for leptin, a key weight control hormone. (See, e.g., Hummel *et al.*, 1966, *Science* 153:1127-1128.) These mice become obese at 3 to 4 weeks of age and develop hyperglycemia. Associated nephropathy includes proteinuria and mesangial expansion with increased mesangial matrix that develops by 5 to 7 months. (See, e.g., Cohen *et al.*, 1995, *J. Clin. Invest.* 95:2338-2345.) In the present experiments, mice were sacrificed at the age of 5 months. Blood glucose levels were determined during the study and at sacrifice, using a colorimetric method based on the glucose oxidase-peroxidase reaction and supplied in a kit form (Glucose Procedure No. 510 kit, Sigma Diagnostics, St. Louis, MO). Following a 24 hour acclimation to metabolic cages, two consecutive 24 hour urine samples were collected. At the

5 end of the collection period, the lower part of the cage including the collection funnel was rinsed with distilled water and the final sample volume recorded. Protein concentration in the urine was measured according to a method for quantifying microgram quantities of protein utilizing protein-dye binding. (See, e.g., Bradford, 1976, *Anal. Biochem.* 72:248-254.)

After anesthesia by an oxygen/ether mixture, the abdominal cavity was opened, a 23  
10 gauge needle was inserted into the aorta and the kidneys were perfused with four (4) ml of ice cold perfusion buffer of (RPMI with 4% BSA) containing 10 mM vanadyl ribonucleoside complex (VRC), an RNase inhibitor (Gibco/BRL, Grand Island, NY). Chilled 0.9% saline was poured over the kidneys during this perfusion. The kidneys were then removed, and the right kidney was frozen in liquid nitrogen for subsequent RNA extraction and Northern analysis. Fine  
15 saggital slices of the left kidney were rapidly obtained. One section was fixed in 3.8 % paraformaldehyde, embedded in parafilm and stained with periodic acid Schiff (PAS) for light microscopic evaluation. The remaining slices were used for glomerular microdissection and reverse transcription and polymerase chain reaction (RT-PCR) of the isolated glomeruli. The methods used were a modification of known methods for determining glomerular mRNA levels. (See, e.g., Peten *et al.*, 1003, *Kidney Int* 39:S55-S58.) Tissue sections were placed in a buffer of  
20 HBSS containing 10 mM VRC, then 50 glomeruli were dissected from each kidney in less than 50 minutes. The glomeruli were next transferred to a PCR tube with 30 µl of rinse buffer (HBSS containing 5 mM DTT and 50 units / ml of human placental ribonuclease inhibitor (Boeringer Mannheim, Indianapolis, IN). Following centrifugation, the supernatant was removed and  
25 microscopically examined for the accidental presence of glomeruli. Seven microliters of a lysis solution (rinse buffer containing 2% Triton X-100) were added, and the samples were stored at -70°C until processed. All of these procedures were carried out at 4°C.

Experimental samples from control and diabetic mice were thawed on ice, then subjected to 2 additional freeze/thaw cycles to lyse the glomeruli. The RT reaction was then carried out  
30 using a cDNA synthesis kit (Boehringer Mannheim), with oligo(dT) as a primer. Reactions containing glomeruli, but without added reverse transcriptase, or without glomeruli, but with reverse transcriptase, served as negative controls. The reaction mixture was incubated for 60



5 minutes at 42°C, then chilled to 4°C for 10 minutes. Samples were then diluted at a ratio of 1:10 in distilled water and frozen at -70°C until PCR was completed.

***Evaluation Of Renal Tissue By Light Microscopy.*** Five to 6 nonconsecutive 6 µm sections per kidney were PAS stained and examined. Mesangial sclerosis was scored on a scale of zero to four (0-4), wherein zero (0) represents no lesion; one (1) represents minimal mesangial expansion; two (2) represents mesangial expansion and/or basement membrane thickening; three (3) represents marked mesangial thickening, some collapsed lumina, and occasional lobule with full sclerosis; and four (4) represents a diffuse collapse of capillary lumina, and sclerosis involving 75% or more of the tuft. A total of 100-150 glomeruli per kidney were scored by an observer blinded as to the origin of the specimens. Only glomerular profiles showing a mesangial region that could be unequivocally evaluated were scored.

***Competitive PCR and Northern blotting.*** All PCR were performed using the GeneAmp DNA amplification kit (Perkin-Elmer Cetus, Norwalk, CT) and a 9600 thermal cycler (Perkin Elmer). For quantitation, a competitive PCR reaction was run using a cDNA mimic. Thirty-eight cycles of replication were used. Five PCR tubes were set up for each sample. Each tube in a series contained a fixed amount of the wild-type cDNA along with decreasing concentrations of the mimic cDNA. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were digitized by scanning densitometry (Howtek, Scanmaster 3+ Densitometer, Hudson, NH) and quantified with image analysis (NIH Image, v. 1.59 from Twilight Clone BBS, Silver Springs, MD). A plot of the ratio of wild type/mimic vs. the reciprocal of the input mutant concentration was constructed and the amount of glomerular cDNA determined from the resulting linear regression. Northern analysis was carried out as previously described, following pulverization of samples in a liquid-nitrogen cooled stainless-steel mortar and homogenization in 1.0 ml of RNA Stat-60 reagent (Tel-Test Inc., Friendswood, TX). Probes for individual mRNAs and the corresponding cDNAs, were labeled with <sup>32</sup>P by random hexamer priming using the Sigma Prime-1 kit (Sigma Chemical). Autoradiograms were digitized by scanning densitometry and quantified as described above.

***Primers, probes, and cDNA mimics.*** Primers for CTGF were designed and synthesized

5 based on conserved sequences between the human and mouse CTGF (fisp 12) gene. The primers, Primer R and Primer F, were as follows: Primer F: 5'-GAG TGG GTG TGT GAC GAG CCC AA G G-3' and Primer R: 5' ATG TCT CCG TAC ATC TTC CTG TAG T-3'. The amplification product was 558 bp in size. The sequence was confirmed by cloning into a PCR script (Invitrogen Corp., Carlsbad, CA). Two clones were sequenced and were identical. A competitive cDNA mimic was produced using a PCR mimic construction kit (K1700-1, Clontech). For each mimic, two composite primers (3' and 5') were first made containing the CTGF target gene sequence, plus a 20-nucleotide stretch designed to hybridize to opposite strands of a heterologous DNA fragment provided in the kit. The desired primer sequences were then incorporated into this fragment during PCR amplification. A dilution of the first PCR reaction was then amplified using only the gene-specific primers. This ensured that all mimic molecules had complete gene-specific sequences. The mimic was then purified by passage through CHROMA SPIN TE-100 columns (Clontech). By this method, the size (200-650 bp) could be adjusted by choosing the appropriate sequences of the generic DNA fragment for the composite primers. The resulting cDNA competes on an equal basis for the same primers in the same reaction. An amplimer of the CTGF mimic was 496 bp in size.

Primers (Primer F and Primer R) for rat fibronectin cDNA were Primer F: 5' TGC CAC TGT TCT CCT ACG TG 3' and Primer R: 5'-ATG CTT TGA CCC TTA CAC GG 3'. A competitive mimic for fibronectin was constructed as described above. Products of amplification were approximately 312 bps (sample) and 474 bps (mimic). Primers for GAPDH (Clontech Laboratories, Palo Alto, CA) produced an amplification fragment of 985 bp. A GAPDH competitive mimic was constructed as described above, and produced a fragment of 604 bp. The cDNA probe for Northern analysis was from a sequence of human CTGF shared by rat and mouse.

***Production of recombinant CTGF and anti-CTGF antibodies.*** Recombinant human CTGF protein (rhCTGF) was generated using a baculoviral expression system. A human CTGF open reading frame of 1047 bp was amplified using primers engineered with BamHI sites immediately flanking the ATG start codon and TGA stop codon (forward primer 5'-GCT CCG CCC GCA

GTG GGA TCC ATG ACC GCC GCC-3'; reverse primer 5' -GGA TCC GGA TCC TCA TGC  
CAT GTC TCC GTA- 3'). A clone designated as Clone DB60R32 was used as a template,  
which contains the entire 2075 bp CTGF cDNA. The amplified product was subcloned into the  
BamHI site of pFastBac1 from Gibco/BRL, analyzed for insert orientation, and verified by  
sequencing of both DNA strands. Generation of recombinant baculovirus containing the CTGF  
cDNA was performed as outlined by Gibco/BRL (pFastBac expression system). Recombinant  
baculovirus stocks were isolated, expanded to high virus titer, and used to infect High Five insect  
cells for expression of CTGF. The recombinant CTGF was purified by heparin sepharose  
affinity chromatography as described previously. Peak fractions containing rhCTGF were  
determined by immunoblotting and Coomassie staining of sodium dodecyl sulfate (SDS)-  
polyacrylamide gels.

Two anti-CTGF antibodies were used. The first, anti-CTGF polyclonal designated,  
pAb839, was prepared by immunizing rabbits with a keyhole limpet hemocyanin-coupled  
synthetic peptide corresponding to amino acids 329-343 (CPG DND IFE SLY YRK) that is  
unique to the carboxy terminus of CTGF. The production of antibody was monitored by ELISA  
with the peptide conjugated to BSA and absorbed to plastic. The anti-CTGF antibodies were  
affinity-purified by passage through a CPG DND IFE SLY YRK-Sepharose peptide column  
using standard protocols (*See, e.g., 1988, A Laboratory Manual*, Cold Springs Harbor, New  
York). Peptide blocking studies confirmed the monospecificity of pAb839 for CTGF in Western  
immunoblotting assays. Western immunoblot analysis also revealed that pAb839 recognized  
CTGF only in a reduced conformation. The second antibody, pIgY3 polyclonal, was raised in  
chickens by immunizing with purified baculovirus-derived full-length rhCTGF protein, and was  
subsequently affinity purified through a rhCTGF-Sepharose column. (*See, e.g., Kothapalli et al.,  
1997, Cell Growth & Differentiation 8:61-68*).

**ELISA.** The amount of specific extracellular matrix components secreted into the culture  
medium was quantified by ELISA, using procedures described in the art. (*See, e.g., Riser et al.,  
1992, J. Clin. Invest. 90:1932-1943*.) It was previously determined in mesangial cell cultures,  
that media containing 0.5-1% FCS was optimal for the recovery of fibronectin and collagen.

5 (See, e.g., Riser *et al.*, 1992, *J. Clin. Invest.*, *supra.*) Experimental samples of culture medium were tested in triplicate. Purified matrix components, diluted in the same medium, were run (0.5- 500 ng / well) as standards. All antisera were tested for specificity before their use by immunoblotting, with and without blocking, using the extracellular matrix standards. Color intensity was measured with a Titertek Multiscan MCC/340 (Flow Laboratories, McLean, VA, and the results analyzed using a curve-fitter computer program (Interactive Microware Inc., State College, PA).

15 An indirect ELISA was used to quantitate CTGF levels in the conditioned media. Microtiter wells were coated with media samples or the rhCTGF standard (50  $\mu$ l/well) for 2 hours at room temperature in a 96-well plate. The wells were washed 4 times with Dulbecco's phosphate buffered saline (D-PBS) and then incubated with pIgY3 antibody at 1.25  $\mu$ g/ml (50  $\mu$ l/well) in a blocking buffer of 1%BSA, 0.05% Tween 20 in D-PBS for 60 minutes. After thorough washing with D-PBS, an HRP-conjugated rabbit anti-chicken IgG (Zymed Laboratories Inc., South San Francisco, CA) was added to all wells at a 1:6400 dilution in blocking buffer for 30 minutes. The substrate, TMB-ELISA (GibcoBRL) was added at room temperature for 15 minutes. The reaction was stopped with 1M sulfuric acid and the color developed measured at 450 nm in an ELISA multiscan spectrophotometer (Molecular Devices, Sunnyvale, CA). The amount of CTGF protein present in samples was determined by using a logarithmic standard curve using serial dilutions of 3 pg to 3 ng /well of rhCTGF standard antigen.

25 ***Heparin sepharose precipitation and immunoblotting.*** To analyze for CTGF protein expression, conditioned media were collected and the heparin-binding proteins precipitated by end-over-end mixing for 4 hours at 4°C with Heparin Sepharose CL-6B beads (Pharmacia, Piscataway, NJ). The beads were washed three times with an ice cold RIPA lysis buffer comprised of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS and 2mM EDTA. The bound proteins were then eluted by boiling in a SDS sample buffer comprised of 62 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol and bromophenol blue for 5 minutes under either non-reducing, or reducing conditions containing 5% mercaptoethanol. 30 The eluted heparin-binding proteins were resolved in 4-20% SDS-polyacrylamide gels and

5 electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) for 2 hours at 140 mA. The filters were blocked with blocking buffer comprised of TTBS; 150 mM NaCl, 50 mM Tris, 0.2% Tween-20, 5% BSA, pH 7.4 for 2 hours at room temperature, and then probed for CTGF by incubation for 40 minutes with an anti-CTGF antibody at 0.5 µg/ml in the blocking buffer. After extensive washing at 37°C, the filters were incubated with either a HRP-  
10 conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL), or a HRP-conjugated rabbit anti-chicken IgG (Zymed, South San Francisco, CA) at a 1:12,000 dilution in the blocking buffer. Immunoreactivity was detected by using a SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

**Additional reagents.** Purified extracellular components used as standards were rat collagen I  
15 (Upstate Biotechnology Inc., Lake Placid, NY) and rat fibronectin (Chemicon International Inc., Temecula, CA). The corresponding antibodies, polyclonal anti-rat collagen I and anti-rat fibronectin were used in ELISA. In preliminary experiments, the polyclonal anti-rat collagen I antibody did not cross-react with fibronectin or laminin, whereas the anti-rat fibronectin antibody did not cross-react with collagen I or laminin. The TGF-β used for stimulation experiments was human TGF-β2 (Celtrix Corporation, Santa Clara, CA). This recombinant cytokine was  
20 produced in Chinese hamster ovary (CHO) cells, then purified by previously reported techniques (See, e.g., 1991, Ogawa *et al.*, 1991, *Methods in Enzymology* 198:317-327). A monoclonal antibody designated as 1.D 11.1, neutralizes TGF-β1, TGF-β2 and TGF-β3 (Genzyme Corporation, Cambridge, MA).

25 **Statistical analysis.** Data was expressed as means ± SEM (Standard Error Mean). For tissue culture data, unless otherwise noted, differences between two groups were evaluated using a paired Student's *t*-test. A paired test was utilized because of the cloned nature of the mesangial cells studied. In the case where results were normalized to corresponding control values, the data was analyzed by a one-sample *t* test with a hypothesized mean of 100% to compare the test group with the control. A paired  
30 two-sample *t* test was used to examine differences between 3 test groups. In both cases, a Holm's test was then applied *post hoc* to adjust for multiple comparisons. (See, e.g., Holm, 1979, *Scan J. Statist.* 6:65-70). For histological data, the mean sclerosis score was calculated in the glomeruli of each kidney

5 and the statistical difference between the diabetic and control groups determined in a non-paired *t* test.

**A. CTGF Is An Important Factor In The Pathogenesis of Renal Diseases, Including Diabetes**

10 The examples provided in the present invention, provides the first evidence demonstrating the production of CTGF protein by glomerular cells and its role as a potentially important factor in the pathogenesis of diabetic glomerulosclerosis. Hyperglycemia and glomerular hypertension are two major, well known, casual factors of diabetic glomerulosclerosis. Prior to the present invention, the role of CTGF in the development of glomerulosclerosis has not been studied. The following examples, demonstrate that CTGF stimulates cultured mesangial cells to produce, deposit, and accumulate extracellular matrix components. The examples also demonstrate that the induction of endogenous CTGF is triggered by increased glucose concentrations, exogenous TGF- $\beta$ , and mechanical strain.

15 As demonstrated in Example 2, CTGF mRNA is expressed in the whole kidney of normal animals, and that its level is high in comparison to the heart and brain, suggesting that endogenously produced CTGF may be involved in the normal turnover of renal extracellular matrix. However, the low levels of constitutive CTGF mRNA expression demonstrated in cultured mesangial cells suggest that this cell type may have a controlling mechanism for CTGF formation different from that in the cells forming the bulk of the renal tissue, *i.e.* tubular epithelial cells. The low expression of CTGF mRNA observed in mesangial cells under unstimulated conditions is associated with an apparent release of small quantities of CTGF protein into the culture medium, see Example 3. The CTGF protein was present as a 36 and 38 kD molecular species. The larger protein is equivalent in size to the full-length CTGF molecule predicted from gene analysis, whereas the smaller peptide may represent a differential N-glycosylation in the CTGF N-terminal half. It was observed that in both insect and mammalian cells, pretreatment with tunicamycin, that inhibits the N-glycosylation of glycoproteins, the larger CTGF band is reduced in its migration to localize with the smaller moiety. These molecular species observed in mesangial cells are similar in size to that secreted by vascular endothelial and fibroblast cells (*See, e.g.,* Bradham et al., 1991, *supra*; Kothapalli et al., 1997,

5 *supra*; and Steffen *et al.*, 1998, *Growth Factors* 15:199-213.) The small amounts of CTGF  
detected in the conditioned medium of mesangial cultures were not the result of low levels of its  
synthesis, but were rather due to the restricted release of the protein into medium. This was  
indicated by the ability of sodium-heparin to dramatically increase the levels of CTGF protein  
measured in the media. (See, Example 3.) These results suggest that as much as 80% of the  
10 CTGF synthesized by mesangial cells remains cell- or matrix-bound. In a quantitative assay it is  
shown that, in the presence heparin, mesangial cells secrete approximately 7 ng of CTGF per 10<sup>6</sup>  
cells in each 24 hour period. (See, Example 3.)

15 Given that CTGF stimulates extracellular matrix accumulation, it was examined whether  
known factors implicated in the development of diabetic glomerulopathy alter CTGF mRNA  
expression. High extracellular glucose concentrations markedly increased the levels of CTGF  
mRNA as well as the production of CTGF protein in mesangial cells. (See, Example 4.) In a  
similar manner, TGF- $\beta$  also upregulates the expression of CTGF mRNA and protein. With  
strong upregulation, as occurred in response to TGF- $\beta$ , there was a marked induction of a small  
molecular weight CTGF species, which according to its size of ~18 kD, is approximately half of  
the full-length CTGF molecule. (See, Example 2 and Figure 6.) The size and properties of this  
small molecular weight CTGF species recovered from a heparin-sepharose column, indicated  
that it contains both the thrombospondin 1 and the C-terminal modules of CTGF. The small  
molecular species demonstrated in mesangial cells following stimulation, may have distinct  
biological activities as compared to the whole molecule. Since TGF- $\beta$  secretion in mesangial  
25 cells is stimulated by increased ambient glucose concentrations, the observed induction of CTGF  
by high glucose may occur indirectly, mediated by the action of TGF- $\beta$ . The neutralization  
studies described in Example 5 demonstrated a direct role for the cytokine in the process, since  
incubation with TGF- $\beta$  antibodies resulted in a complete blockade of CTGF stimulation. (See,  
Figure 8.)

30 Cyclic mechanical strain was also examined as a possible regulatory element in  
CTGF expression. The results demonstrated that stretching is a potent stimulus for the  
upregulation of CTGF mRNA levels (See, Example 7 and Figure 10). The rapid induction of

CTGF mRNA following stretch suggests that TGF- $\beta$  production and/or activity may not be required to mediate the initial effects of mechanical strain. Cyclic strain induces TGF- $\beta$ 1 synthesis and activation, but this effect is only evident after 48-72 hours of mechanical stimulation. (See, e.g., Riser *et al.*, 1992, *Am. J. Path.*, *supra*). These studies also demonstrated that TGF- $\beta$  and CTGF are able to autoinduce their own expression in mesangial cells. (See, Example 3 and Figure 3.) This autoinducing action of CTGF is the first time that such action has been observed for CTGF. Furthermore, this action appears to be selective, since exogenous CTGF has no effect on TGF- $\beta$  transcript levels. (See, Figure 3.) These findings suggested that once stimulated by TGF- $\beta$ , CTGF mRNA levels in mesangial cells may remain elevated even in the absence of additional TGF- $\beta$  activity resulting in a continued enhancement of extracellular matrix synthesis and deposition, which may explain the prevalent inability to totally block extracellular matrix production in mesangial cells and in the mesangium by TGF- $\beta$  neutralization. (See, e.g., Border *et al.*, 1990, *supra*; Sharma *et al.*, 1996, *supra*; and Ziyadeh *et al.*, 1994, *supra*.)

Quantitative glomerular expression of CTGF mRNA in db/db mice (See, Example 10), demonstrated that CTGF action is a factor in the initiation of glomerular extracellular matrix deposition in diabetes. While CTGF mRNA is expressed in normal glomeruli, the levels are dramatically upregulated by 28-fold, after a short period of diabetes and before the onset of overt glomerular disease (See, Example 10). As demonstrated in these examples, CTGF mRNA upregulation occurred at a time when glomerular fibronectin mRNA levels were increased.

However, the glomerular mesangial expansion was minimal and proteinuria insignificant. As compared to glomeruli, the much lower upregulation of CTGF observed in the whole kidney as demonstrated in Example 10 shows that the CTGF is, at least in the early phases of nephropathy, primarily involved in the induction of the glomerular alterations. However, in the more advanced stages of diabetic nephropathy, CTGF may be an important inducer of tubulointerstitial disease.

In summary, the following examples essentially demonstrate that, in addition to enhanced glomerular TGF- $\beta$  expression, CTGF upregulation is an important factor in the excess deposition of the extracellular matrix by mesangial cells. This CTGF upregulation is driven by a



combination of high glucose concentrations and cellular mechanical strain via pathways that are both dependent and independent of TGF- $\beta$  stimulation.

## **B. Experimental Data Demonstrating Nexus Between The Presence Of CTGF And The Onset And Progression Of Renal Disorders, Including Diabetes**

### ***Example 1: CTGF-Induced Changes In Extracellular Matrix Production Of***

**Mesangial Cells.** To determine the effects of exogenous CTGF on mesangial cell production of the extracellular matrix, serum-depleted cells were exposed for 48 hours to media containing 20 ng/ml of rhCTGF. For comparison purposes, additional cultures were incubated in media without exogenous CTGF, but containing either 2 ng/ml of TGF- $\beta$ , or 20 mM glucose. As anticipated, exogenous TGF- $\beta$  and the high glucose concentration increased the amount of secreted fibronectin by 23 and 30%, respectively, over that of controls as shown in Figure 1A. The presence of exogenous CTGF in the media also effectively stimulated fibronectin secretion by 45%. Like fibronectin, the quantity of secreted collagen type I was also increased by 64% CTGF, as well as by 50% TGF- $\beta$  or 22% high glucose as shown in Figure 1B.

### ***Example 2: Renal And Mesangial Cell CTGF Expression: Regulation By TGF- $\beta$ .***

It was determined whether CTGF mRNA was expressed by cultured rat mesangial cells, and the results were compared to those from whole kidney. Northern analysis demonstrated a single 2.4 kb CTGF transcript in mesangial cells and whole kidney, but in contrast no detectable message was evident in cultured kidney fibroblasts as demonstrated in Figure 2. When compared to other tissues, the most abundant expression was in the kidney, being approximately 20-fold higher than in the brain.

To determine if TGF- $\beta$  was a regulatory factor in mesangial cell expression of CTGF message, cells were serum-depleted, exposed to 2 ng/ml of TGF- $\beta$  for 24 hours, the mRNA was then probed. Changes in TGF- $\beta$  transcript levels were also monitored. Exogenous TGF- $\beta$  exposure increased the expression of CTGF mRNA greater than 4-fold as shown in Figure 3A and Figure 3B, whereas TGF- $\beta$  mRNA increased 80% (see, Figure 3A and Figure 3C). To determine whether CTGF was capable of regulating its own expression, or that of TGF- $\beta$ , mesangial cells were also exposed to 20 ng/ml of rhCTGF. As shown in Figure 3A and Figure 3C, this treatment did not alter the level of TGF- $\beta$  mRNA, but in contrast, strongly autoinduced CTGF message as demonstrated in Figure 3A and Figure

5 3B.

To demonstrate whether low CTGF mRNA expression in unstimulated mesangial cells was associated with a detectable production of the corresponding protein, and to determine the effects of TGF- $\beta$ , cells were serum depleted and then cultured for an additional 24 hours in fresh maintenance medium in the presence or absence of 2 ng/ml exogenous TGF- $\beta$ . The conditioned medium was subsequently heparin-sulfate precipitated and analyzed by immunoblotting using two different anti-CTGF antibodies. Immunoblotting with pIgY3 antibody, raised against the full-length rhCTGF, demonstrated that under basal, unstimulated conditions mesangial cells secreted very small amounts of CTGF (*see*, Figure 4A). However, upon exposure to TGF- $\beta$ , the secretion of CTGF protein was markedly stimulated. The predominant product detected in these cultures migrated to the same position as the recombinant standard. Immunoblotting of the same samples with the pAb839 antibody, raised against a 15 amino acid sequence unique to CTGF, confirmed the identity of the protein detected (*see*, Figure 4B).

**Example 3: Detection Of CTGF In Mesangial Cells.** The CTGF protein detected in mesangial cell cultures above represents free molecules present in the media. The existence of a heparin binding domain within CTGF suggests that a substantial portion of the synthesized and released protein exists bound to proteoglycans, or to fibronectin, present on the cell surface or in the extracellular matrix. To ascertain whether this was the case, and to determine the time course for appearance of CTGF in the extracellular environment under unstimulated conditions, mesangial cell cultures were serum-deprived and then fresh maintenance media containing 50  $\mu$ g/ml of sodium heparin was added. Conditioned media were collected after defined incubation periods and the majority of the sample pooled and heparin-sulfate precipitated. Immunoblotting of the 4 hour samples produced faint CTGF bands at approximately 36 and 39 kD (*see*, Figure 5A). The intensities of these bands increased sharply by 24 hours and remained elevated throughout the 72 hour incubation period. At 48 and 72 hours, when the full-length CTGF bands were intense, a faint additional band with an electrophoretic mobility of

5 approximately 20 kD could also be detected. As previously demonstrated, in the absence of sodium heparin, the CTGF present in the media was barely detectable, suggesting that the majority of CTGF protein produced was bound to the cell and/or substrate. Because immunoblotting is largely a qualitative assay, individual supernatants were also evaluated by ELISA prior to their pooling and precipitation, and the results were expressed on a per cell basis. 10 This highly quantitative assay demonstrated a time dependent increase in CTGF, with approximately 7 ng / 10<sup>6</sup> cells being secreted in a 24-hour period as shown in Figure 5B. The amount of CTGF secreted in the medium during the total 72-hour period was reduced to 20% in the absence of heparin.

15 In a subsequent experiment, the regulation of secreted CTGF by TGF- $\beta$  was reexamined, in the presence of heparin. Accordingly, mesangial cells were serum-deprived, then incubated for 48 hours in a maintenance media containing 50  $\mu$ g/ml of sodium heparin and 2 ng /ml TGF- $\beta$ . Immunoblotting of pooled, precipitated media samples indicated that TGF- $\beta$  markedly increased the secretion of full-length (36-39 kDa) CTGF as demonstrated in Figure 6A. However, even more pronounced was the induction of the molecule(s) appearing at 18-20 kDa. 20 This smaller moiety corresponds in size to half of the full-length CTGF molecule. Quantitative analysis by ELISA of the individual samples, prior to being pooled and precipitated for immunoblotting, demonstrated a 2.5-fold enhancement of total secreted CTGF in response to TGF- $\beta$  treatment (*see*, Figure 6B).

#### ***Example 4: Mesangial Cell Expression Of CTGF: Regulation By Glucose***

25 ***Concentrations.*** To determine if CTGF expression might also be altered by the ambient concentration of glucose, mesangial cell cultures continuously grown in 5 mM glucose were incubated for 14 days in growth media containing 35 mM glucose. The time was chosen because previous studies demonstrated that this period was required for the full induction of ECM protein production. (*See, e.g., 1997, J. Am. Soc. Nephrology, supra.*) As shown in Figure 7, mesangial 30 cells grown in medium containing 5 mM glucose concentration demonstrated minimal levels of CTGF message. However, following long-term exposure to an increased glucose concentration, mesangial cell transcripts for CTGF were markedly upregulated , reaching a 7-fold level above

5 control, as determined by quantitative image analysis as depicted in Figure 7.

To examine the effects of high glucose exposure on the secretion of CTGF protein, serum-deprived cultures that shortened the exposure time to 48 hours, and included sodium heparin in the medium were used. This protocol allowed a comparison to the effects of TGF- $\beta$ . Immunoblotting of pooled and precipitated media samples indicated that exposure to 20 mM of glucose increased the amount of CTGF secreted as shown in Figure 6A. Interestingly, however, this stimulation appeared to be limited to the full-length molecule only. Quantitation of secreted CTGF protein by ELISA, prior to pooling and precipitation demonstrated a 2-fold induction by high extracellular glucose levels as demonstrated in Figure 6B, which is an increase similar to that induced by TGF- $\beta$ , under the experimental conditions selected. To determine if the observed increase in CTGF could be due to an osmolar effect, the experiments were repeated using a mannitol. Under these conditions, there was no induction of CTGF released as measured by ELISA (5 mM glucose,  $2.39 \pm 0.28$  ng /  $10^6$  cells; 5 mM glucose plus 15 mM mannitol,  $1.94 \pm 0.32$ ), and no change in the distribution of CTGF forms secreted as determined by immunoblotting.

**Example 5: TGF- $\beta$  Block Of High Glucose-Induced CTGF Production.** To determine if TGF- $\beta$  is responsible for CTGF production by mesangial cells in the presence of high glucose, mesangial cells were cultured for 14 days in the presence of either 5 mM glucose or 20 mM glucose, and were seeded and grown under the same glucose conditions for an additional 8 day period. On day 4, the cultures were serum-deprived, and half received 20  $\mu$ g/ml of an antibody that neutralizes TGF- $\beta$ 1, 2 and 3 activity. Fresh antibody was added daily, and the media was replaced 24 hours prior to collection. Measurement of CTGF secretion by ELISA demonstrated a stimulatory effect of high glucose as depicted in Figure 8. However, neutralization of TGF- $\beta$  activity in these cultures blocked the induction of CTGF by high glucose. While the constitutive secretion of CTGF in the presence of normal concentrations of glucose also appeared somewhat reduced by the presence of a TGF- $\beta$  antibody, this change was not statistically significant ( $p = 0.09$ ). Also non-significant ( $p = 0.075$ ) was the difference in

5 CTGF levels in normal glucose- and high glucose-treated cells when TGF- $\beta$  was neutralized (see, Figure 8).

10 **Example 6: Glucose transporter expression and CTGF production.** Mesangial cells transduced with the human glucose transporter 1 (GLUT1) gene producing a line designated, MCGT1, demonstrated a 10-fold increase in GLUT1 protein, a 5-fold increase in glucose uptake and a 2-3 fold increase in the synthesis of collagen types I and IV, fibronectin and laminin, as compared to a control mesangial cell line, designated MCLacZ, and transduced with the bacterial  $\beta$ -galactosidase gene. These cell lines were used to demonstrate that the increase in intracellular glucose, rather than simply the extracellular glucose concentration *per se*, is the major determinant of exaggerated extracellular matrix formation by mesangial cells in culture. (See, e.g., Helig *et al.*, 1995, *supra*.) To determine if CTGF is also increased in this *in vitro* model of diabetes, MCCT1 and MCLacZ cells were seeded and grown for 48 hours in RPMI with 20% NuSerum, 8 mM glucose. Cells were then washed twice in serum-free media and fresh RPMI containing 1% FCS added. Conditioned media were collected 24 hours later and CTGF protein levels were determined by ELISA. Approximately 80 ng/10<sup>6</sup> cells of CTGF was detected in the media of the control MCLacZ cultures whereas the level nearly doubled (147 ng/10<sup>6</sup> cells) in MCGT1 cultures as shown in Figure 9.

25 **Example 7: Mesangial Cell Expression Of CTGF: Regulation By Cyclic Mechanical Strain.** To determine if cyclic mechanical strain was also a factor capable of altering mesangial cell expression of CTGF, cells were seeded into collagen-coated flexible-bottom plates, then after overnight incubation, and subjected to either stretch or maintained under static conditions. Stretching was set at 3 cycles per minute and 19% maximum elongation using the computer-controlled system previously described in Riser *et al.*, 1992, *J. Clin. Invest.* 90:1932-1943 and Riser *et al.*, 1996, *Am. J. Path.* 148:1915-1923. This degree of stretching was chosen to approximate the mechanical force experienced by mesangial cells *in vivo*. (See, e.g., 30 1997, Cartes *et al.*, *supra*.)

At the designated periods, the cells were lysed and total RNAs extracted and probed for CTGF transcripts. Cyclic stretching induced a rapid and marked increase in CTGF

message as shown in Figure 9. Quantitative image analysis of the Northern blot showed that levels of CTGF mRNA increased more than 2-fold by 4 hours and remained elevated at this level after 8 hours of stretching. Additional experiments demonstrated that CTGF transcripts were significantly increased even after 48 hours of stretch.

***Example 8: Blockade Of Stimulated Collagen Production By Anti-CTGF***

**Antibody.** Mesangial cells were grown for 4 days in RPMI medium with 20% NuSer, the medium was replaced with one containing 1% FCS (serum-deprived conditions). and 0 or 5 ng/ml of TGF- $\beta$ 2. Half of the cultures received anti-CTGF antibody (goat affinity purified, pGAP) and the other half received non-immune goat IgG. Fresh antibody was added daily, and the media was replaced 24 hours prior to collection. Media from individual wells were tested by ELISA. As shown in Figure 11, treatment with anti-CTGF antibody did not alter the production of baseline collagen, but completely blocked the increased production due to TGF- $\beta$ . There was no significant difference between the amount of collagen produced in unstimulated cultures as compared to that of cultures stimulated by TGF- $\beta$ , but treated by anti-CTGF antibody.

***Example 9: Blockade Of Stimulated Mesangial Cell Proliferation By Anti-***

**CTGF Antibody.** Mesangial cells were grown for four days in RPMI medium with 20% NuSer, the medium was replaced with one containing 1% FCS (serum-deprived conditions) and 0 or 5 ng/ml of TGF- $\beta$ 2. Half of the cultures received anti-CTGF antibody (goat affinity purified, pGAP) and the other half non-immune goat IgG. Fresh antibody was added daily, and the media was replaced 24 hours prior to collection. Media from individual wells were tested by ELISA.

As shown in Figure 12, TGF- $\beta$  treatment significantly induced (87%) mesangial cell proliferation. Treatment with anti-CTGF antibody significantly reduced (approximately 50%), the induction of cell proliferation. The same antibody treatment had no effect on basal proliferation, *i.e.* under unstimulated conditions.

***Example 10: CTGF Expression In Experimental Diabetic Nephropathy.*** To

determine if CTGF is upregulated in early diabetic nephropathy, studies were carried out on diabetic *db/db* mice, and the results compared to those from age-matched nondiabetic *db/m* littermates. At 5 months of age, approximately 3.5 months after the onset of diabetes, animals

5 were evaluated for blood glucose levels, total weight, proteinuria and mesangial expansion. At the time of sacrifice, mean blood glucose levels, as well as body weights, were significantly greater in the *db/db* animals as shown in the following Table 1.

Table 1

	Control db/m	Diabetic db/db	
Blood Glucose	142 ± 19.0 mg / dL, n = 8	485 ± 58.0, n = 6	P < 0.001
Weight	29.2 ± 1.00 g, n = 8	43.1 ± 7.30, n = 6	P < 0.001
Proteinuria	2.32 ± 1.07 mg / 24 h, n = 10	2.78 ± 0.93, n = 9	P = 0.330
Glomerular Sclerosis	0.101 ± 0.048, n = 17	0.649 ± 0.369, n = 6	P = 0.003

10 As shown in Figure 13, inspection of the renal tissue by light microscopy demonstrated that the diabetic animals exhibited noticeable, but minimal, glomerular changes consistent with early diabetic glomerulosclerosis, *i.e.* mild mesangial matrix expansion without apparent tubulointerstitial disease. In addition, Table 1 shows that the level of proteinuria was not significantly greater than in controls. Semiquantitative analysis of the glomerular changes demonstrated that the observed mesangial expansion in the diabetic animals was indeed consistent, but of mild intensity. A value of zero (0) represents no lesion and a value of one (1) represents minimal mesangial expansion in the majority of glomeruli, without basement membrane thickening.

20 Northern analysis of whole kidney RNAs indicated that the CTGF message levels were markedly increased in 4 out of 5 diabetic mice as shown in Figure 14A. These changes were mirrored by parallel changes in fibronectin transcript levels. Quantitation of results yielded a mean 103% increase in CTGF expression while fibronectin levels were 80% greater than in the controls as shown in Figure 14B and Figure 14C. Moreover, transcript levels were detected in competitive RT-PCR for CTGF mRNA in a single sample from diabetic mouse glomeruli as

5 compared to the control GAPDH sample as depicted in Figure 15A and Figure 15B. Analysis of  
microdissected glomeruli identified multiple animals (5 diabetic and 5 control groups), that by a  
competitive and quantitative RT-PCR method (described above), identified a low, but  
measurable, transcript level of CTGF in the glomeruli of control animals. (See, Figure 16.) In  
mice with diabetes, the level of CTGF was dramatically increased by 27-fold. (See, Figure 16.)  
10 The upregulation of glomerular CTGF mRNA was accompanied by a nearly 5-fold increase in  
the amount of fibronectin mRNA. These large differences were not due to dissimilar glomerular  
size resulting from diabetic hypertrophy, since the level of GAPDH message was not  
significantly increased in diabetic animals as compared to controls (control,  $1.39 \pm 0.524 \times 10^{-1}$   
attomoles / glomerulus; diabetic  $2.59 \pm 0.307$ ;  $P > 0.05$ ). Therefore, a dramatic increase in  
15 CTGF expression was documented at a time when changes in the kidney were minimal.

### **C. Detection of CTGF In Samples As An Indicator of Renal Diabetes Associated Disorders**

*Example 11: The Presence And Stability Of Urinary CTGF.* To examine  
whether CTGF protein was secreted in the urine, and to examine the stability of the CTGF  
molecule after its secretion in the urine, samples of urine were collected from a healthy donor  
and divided into five, 25-ml aliquots. Various amounts of rhCTGF, ranging from 25 to 750 ng  
was added to four of the five aliquots (the “spiked samples”). The fifth aliquot served as a  
control, receiving no added CTGF (the “unspiked sample”). All samples were frozen, and stored  
at  $-70^{\circ}\text{C}$ , then later thawed and clarified by centrifugation. Following heparin sepharose  
25 quantittative extraction of samples, immunoblotting was performed using CTGF specific  
antibody as indicated in Figure 17. The results identified the presence of a scarcely detectable  
level of CTGF secretion in the urine of the unspiked sample. Further, the stability of the CTGF  
protein in the urine was demonstrated by the progressive increase in CTGF recovered in the  
spiked samples. Comparison of sample lanes with that containing freshly added rhCTGF (35 ng)  
30 indicated that CTGF was largely, if not entirely, preserved.



5                   **Example 12: CTGF In The Urine Of Renal Patients.** The quantity and/or  
molecular form of CTGF present in the urine that might be altered in patients with established  
nephropathy, including that associated with diabetes, was investigated. Urine samples from 8  
ambulatory patients being treated for a variety of kidney diseases, of which 3 had a history of  
diabetes, were collected and frozen during routine visits (Nephrology and Hypertension Clinic,  
10   Henry Ford Hospital). Similarly, samples were also obtained from 3 normal healthy volunteers  
with no history of kidney disease. All samples were later batch-thawed and processed above.  
CTGF was detected in 1 of 3 normal volunteers, and in all patient samples as shown in Figure  
18. Immunoreactive CTGF appeared in 3 different molecular forms. A CTGF band (doublet)  
was present in 1 control sample and 4 of 8 patients samples. Interestingly, a large molecular  
15   weight band, approximately 200kDa, was present in every patient sample, appearing only as a  
very faint band in a single control sample. This large band likely represents CTGF in complex  
with a second, unknown urinary protein. Even more intriguing was a unique small CTGF  
fragment, approximately 9-12 kDa. This smaller moiety appears to be equivalent to the heparin  
binding C-terminal quarter fragment of CTGF, and was present in the urine of all 3 diabetic  
20   patients, but not present in nondiabetic patients or healthy controls. Interestingly, this product  
may compare to the CTGF fragment produced by mesangial cells in culture when stimulated by  
high glucose concentrations of TGF- $\beta$ .

In a separate experiment, CTGF in human urine samples was measure by ELISA, and  
presented in the following Table 2.

Table 2

Patient Population			
Patient Number	Healthy Control	Kidney Disease	Diabetic: No Kidney Disease
1	0.81	0.36	0.68
2	0.43	1.22	7.12
3	0.84	5.46	5.68
4	0.67	2.53	0.40
5		5.72	0.56
6		0.78	3.84
7		3.47	
<b>Mean CTGF</b>	0.69	2.79	3.05
<b>± SE</b>	0.10	0.83	1.12

Table 2 compares the amounts of CTGF detected in healthy volunteers to patients with kidney disease (in some cases associated with diabetes), or to patients with 5 to 10 years with diabetes, but without kidney disease. Each group had 4 to 7 samples from different individuals. Amounts of CTGF/ml were first determined by ELISA, comparing sample values to a standard curve using serial dilutions of a known quantity of rhCTGF. To standardize results (*i.e.* to account for variation in the production of urine), amounts of CTGF (CTGF/ml) were then divided by the urine creatinine from the same patient, determined from the same urine.

The results demonstrate that healthy individuals demonstrate consistently low levels of urinary CTGF. However, among those with kidney disease the mean level of CTGF increased 4-fold. In those patients with diabetes, but as yet undiagnosed kidney disease, there was a similar 4.4 -fold increase. It was expected that because only approximately 40% of those with diabetes will go on to develop nephropathy, a similar percent of patients would exhibit increased CTGF levels. Interestingly, of the 6 diabetic patients tested, 3, or 50%, demonstrated clearly elevated CTGF levels. The remaining patients appeared to have values similar to those of the healthy volunteers.

5           Various modifications and variations of the described methods and systems of the  
invention will be apparent to those skilled in the art without departing from the scope and spirit  
of the invention. Although the invention has been described in connection with specific preferred  
embodiments, it should be understood that the invention as claimed should not be unduly limited  
to such specific embodiments. Indeed, various modifications of the described modes for carrying  
10 out the invention which are obvious to those skilled in molecular biology or related fields are  
intended to be within the scope of the following claims. All patents, publications, and other  
references cited herein are incorporated by reference herein in their entirety.

**CLAIMS**

What is claimed is:

1. A method of treating or preventing fibrosis, the method comprising administering to a subject in need an effective amount of an agent that modulates, regulates, or inhibits the expression or activity of CTGF or fragments thereof.

2. A method of treating or preventing a renal disorder associated with overproduction of extracellular matrix, the method comprising administering to a subject in need an effective amount of an agent that modulates, regulates, or inhibits the expression or activity of CTGF or fragments thereof.

3. The method of claim 2, wherein the agent is an antibody which specifically binds to CTGF or fragments thereof.

4. The method of claim 2, wherein the agent is an antisense oligonucleotide having a sequence which binds with a sequence encoding CTGF or fragments thereof.

5. The method of claim 2, wherein the agent is a small molecule.

6. The method of claim 2, wherein the agent is a compound that blocks binding interactions involved in the signal transduction pathway of CTGF, thereby modulating or inhibiting CTGF activity.

7. The method of claim 2, wherein the agent is a molecule that interferes with or blocks post-translational modification of CTGF.

5           8.       The method of claim 2, wherein the agent is a molecule that blocks the activation  
of a precursor of CTGF.

          9.       The method of claim 2, wherein the renal disorder is diabetes.

10          10.      The method of claim 2, wherein the renal disorder is hypertension.

          11.      The method of claim 2, wherein the extracellular matrix is comprised of collagen.

15          12.      A method of treating or preventing diabetes, the method comprising administering  
to a subject in need a therapeutically effective amount of insulin and a therapeutically effective  
amount of an agent that modulates, regulates, or inhibits expression and activity of CTGF or  
fragments thereof.

20          13.      A pharmaceutical composition comprising an effective amount of an agent that  
modulates, regulates, or inhibits the expression or activity of CTGF.

25          14.      A method for diagnosing a renal disorder characterized by the overproduction of  
extracellular matrix, or identifying a predisposition or susceptibility to a renal disorder  
characterized by the overproduction of extracellular matrix, in a subject, the method comprising:

- (a)     obtaining a sample from the subject
- (b)     detecting the level of CTGF in the sample;
- (c)     comparing the level of CTGF in the sample to a standard level of CTGF.

30          15.      The method of claim 14, wherein the renal disorder is diabetes.

          16.      The method of claim 14, wherein the sample from the subject is a urine sample.

5           17.     A diagnostic kit for use in diagnosing a renal disorder renal disorder characterized by the overproduction of extracellular matrix, or identifying a predisposition or susceptibility to a renal disorder characterized by the overproduction of extracellular matrixcomprising:

- (a)     a means for detecting the level of CTGF in a sample; and
- (b)     a means for measuring the level of CTGF in the sample.

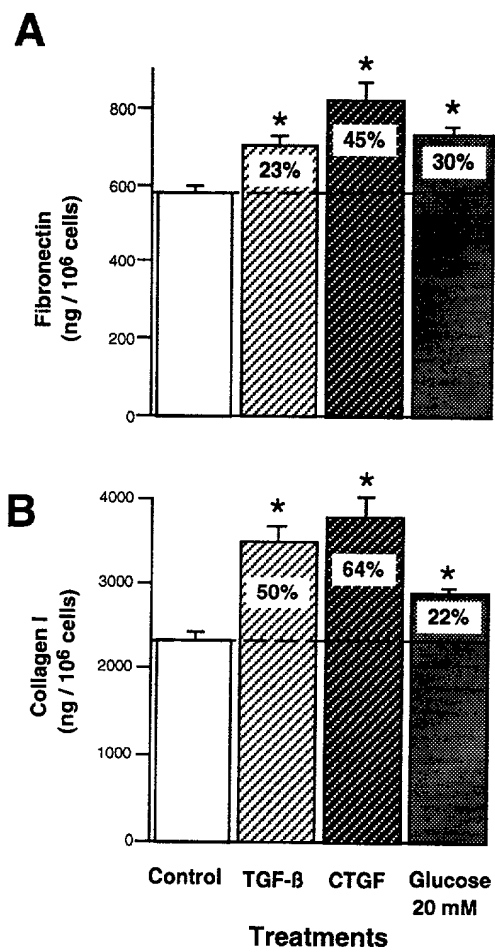
10

18.     The diagnostic kit of claim 17, wherein the sample is a urine sample.

## ABSTRACT

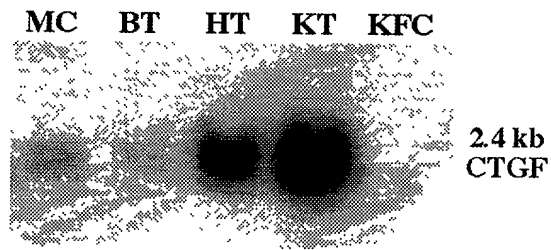
The present invention relates to methods for detecting, preventing, and treating the progression of pathologies characterized by an accumulation of the extracellular matrix components by providing an agent to regulate, inhibit, or modulate the activity of Connective Tissue Growth Factor (CTGF). The method of the present invention is directed to treating kidney fibrosis and associated renal disorders, in particular, complications associated with diabetes and hypertension.

Figure 1



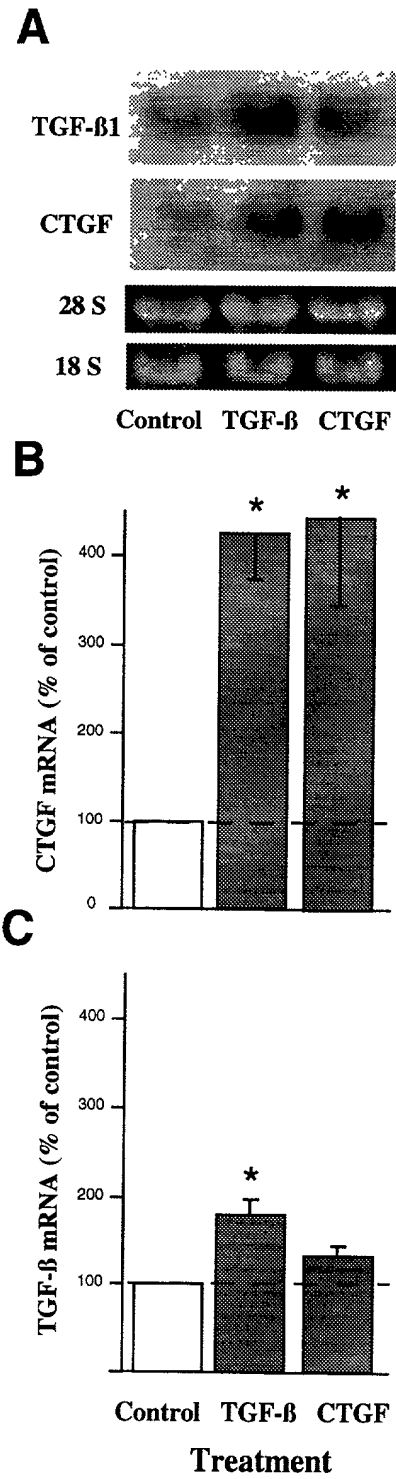


**Figure 2**

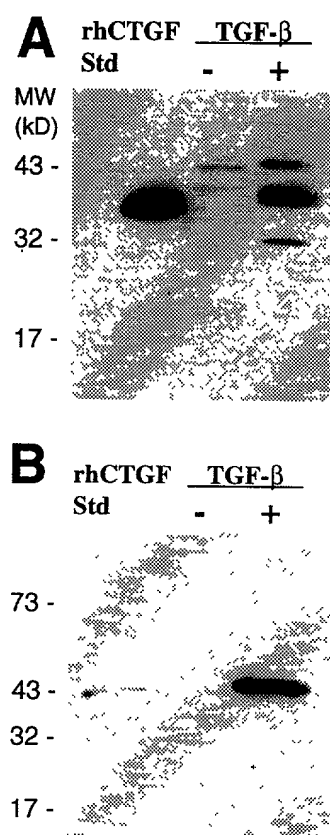


*F*

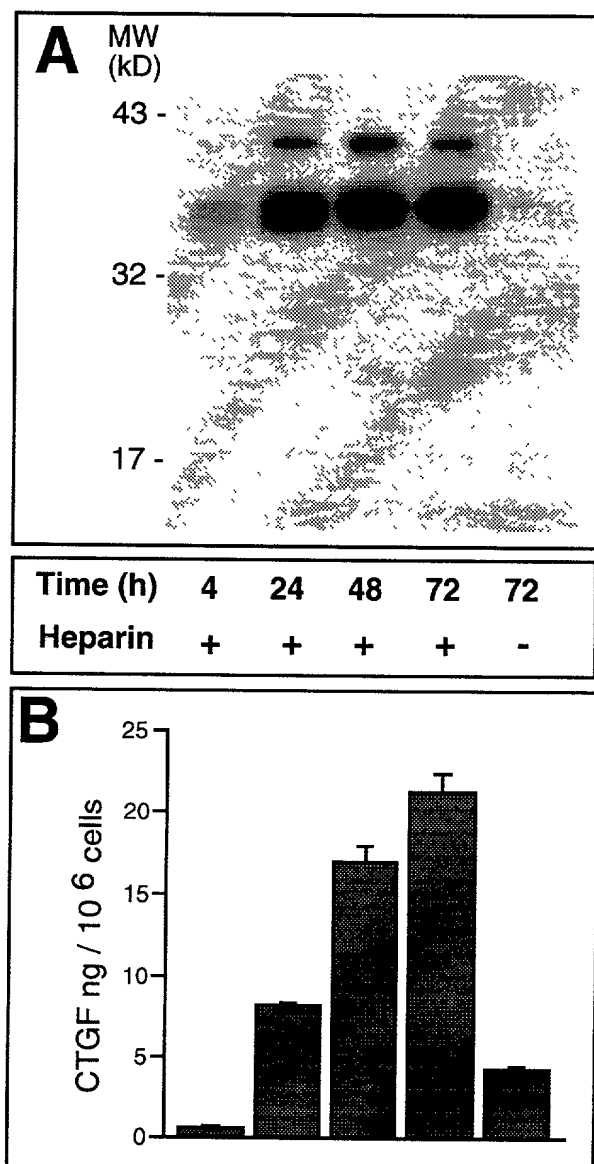
Figure 3



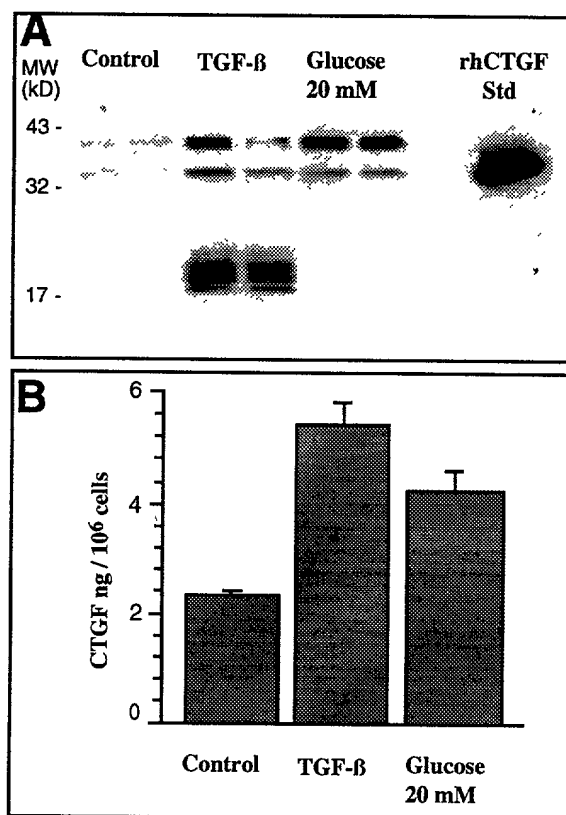
**Figure 4**



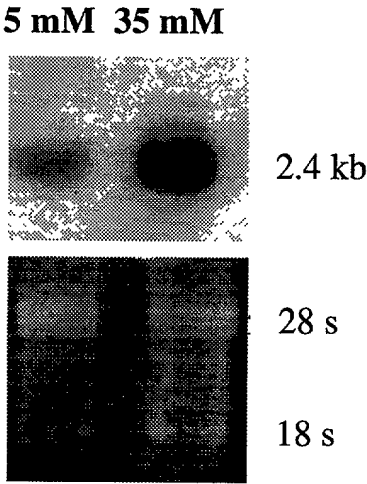
**Figure 5**



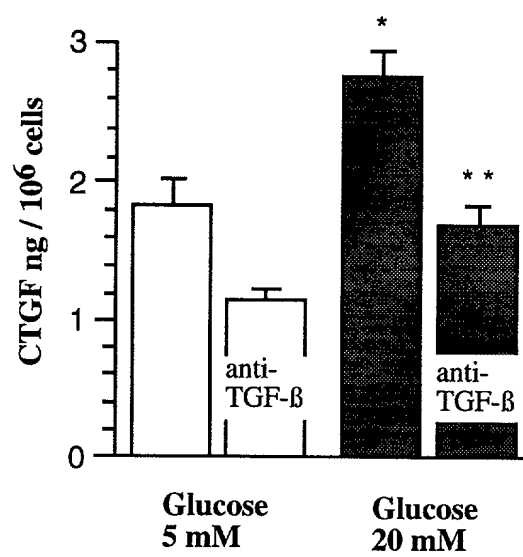
**Figure 6**



**Figure 7**



**Figure 8**



**Figure 9**

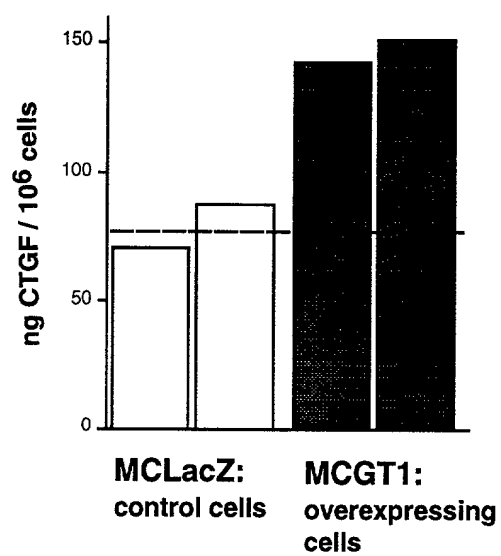




Figure 10

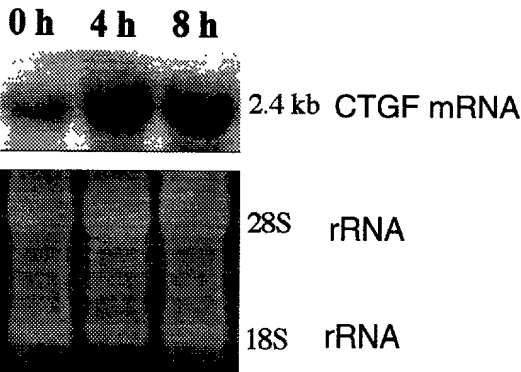


Figure 11

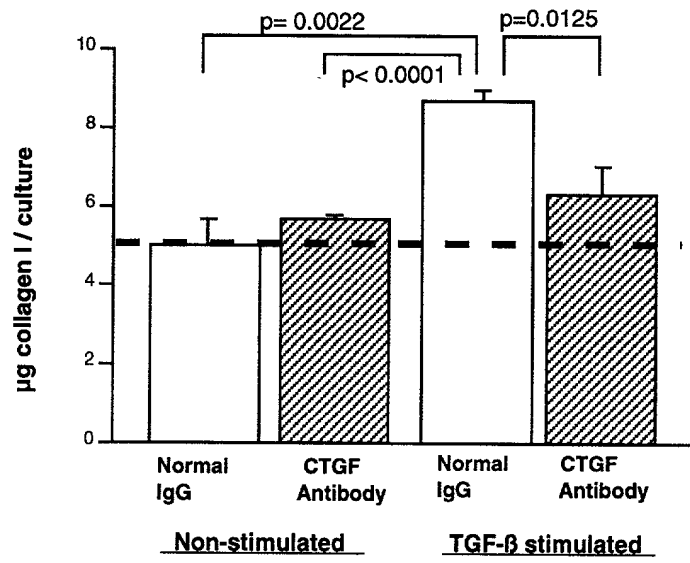


Figure 12

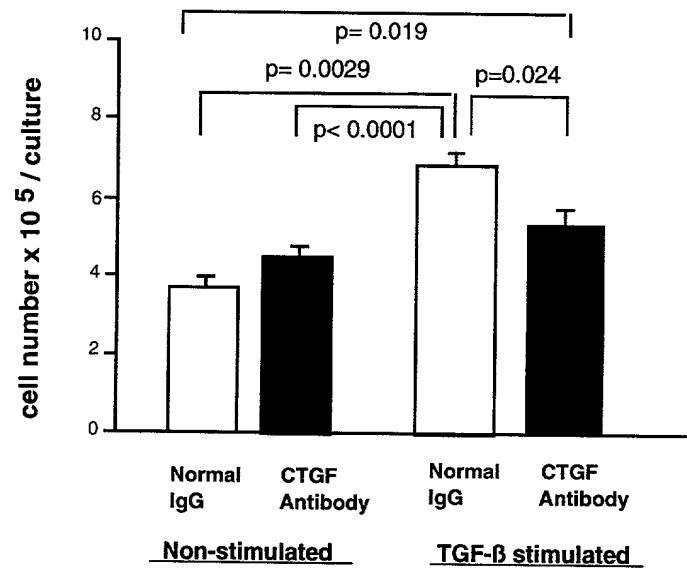


Figure 13

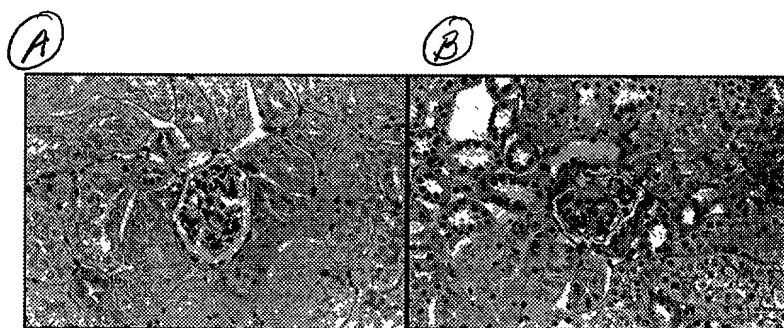


Figure 14

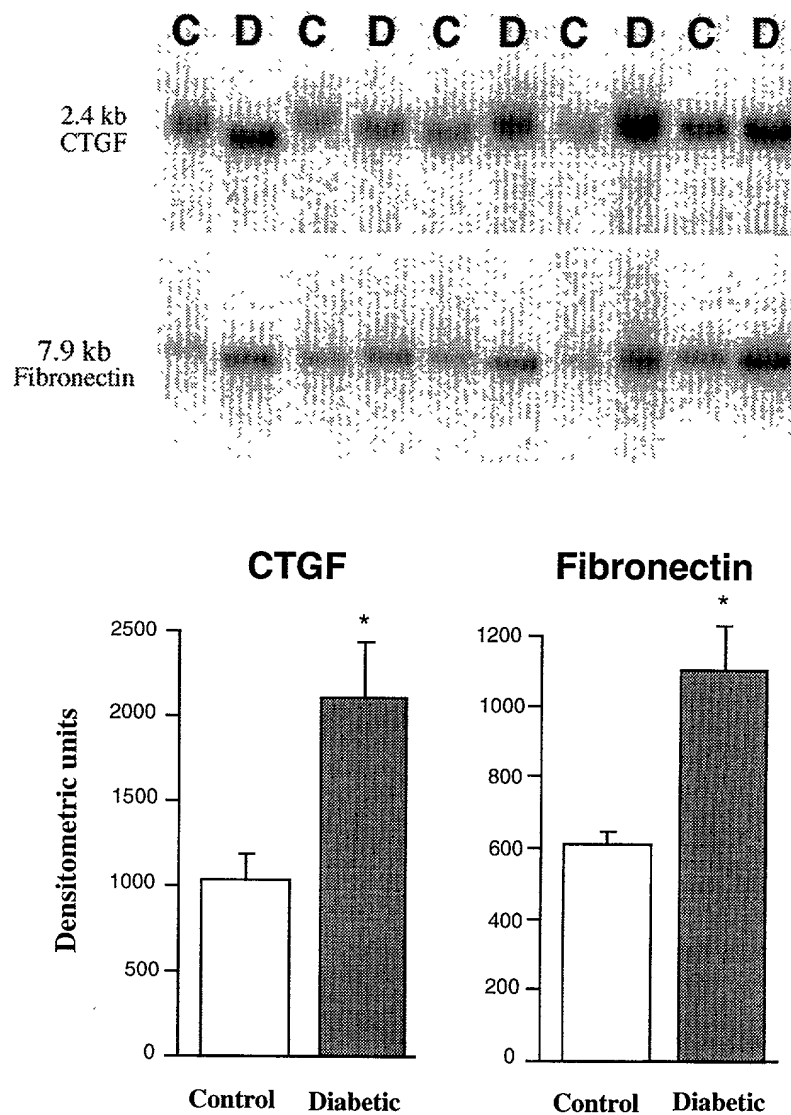


Figure 15

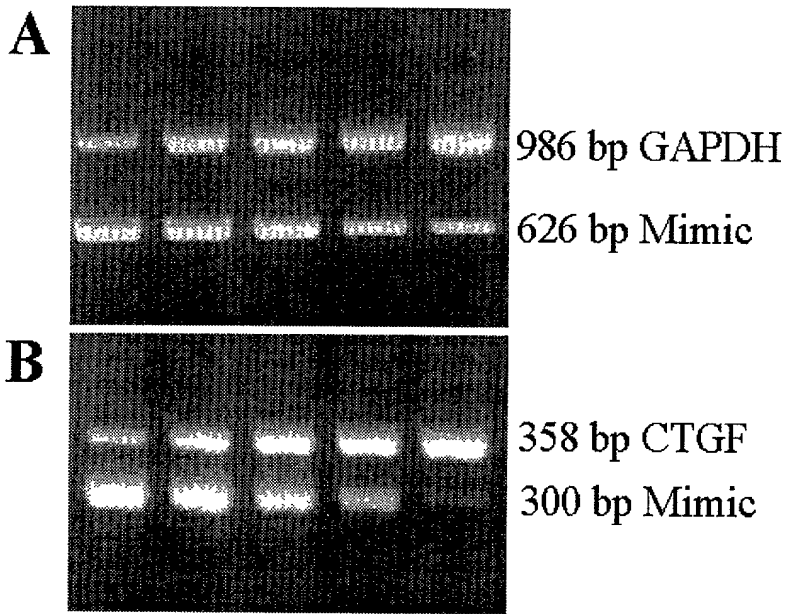


Figure 16

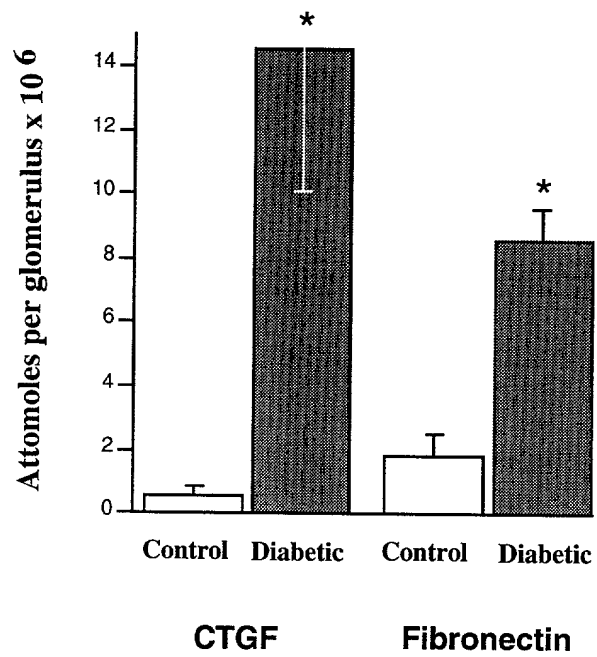


Figure 17

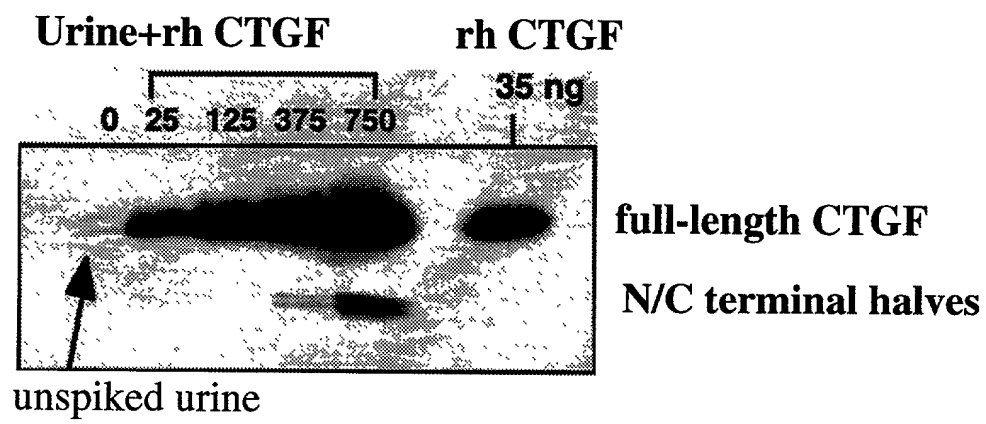
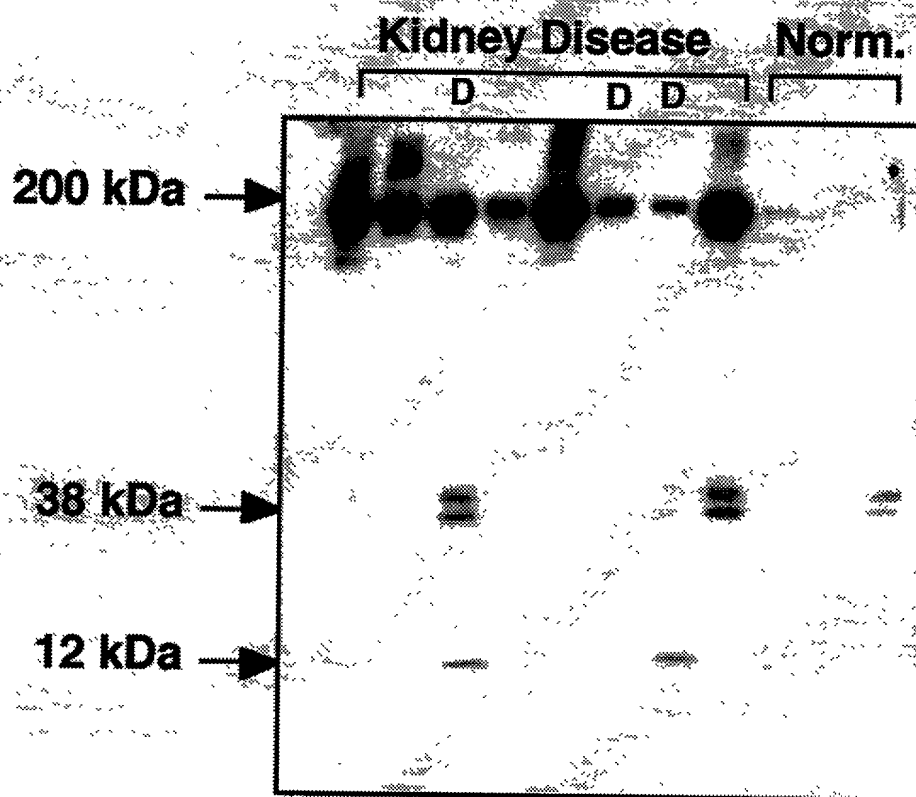




Figure 18



Docket No.  
FG0810

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METHODS FOR DETECTING, PREVENTING, AND TREATING RENAL DISORDERS BY MODULATING, REGULATING, AND INHIBITING CONNECTIVE TISSUE GROWTH FACTOR**

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application No. or PCT International Application Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

\_\_\_\_\_  
(Number)

\_\_\_\_\_  
(Country)

\_\_\_\_\_  
(Day/Month/Year Filed)

☐

\_\_\_\_\_  
(Number)

\_\_\_\_\_  
(Country)

\_\_\_\_\_  
(Day/Month/Year Filed)

☐

\_\_\_\_\_  
(Number)

\_\_\_\_\_  
(Country)

\_\_\_\_\_  
(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/099,471

September 8, 1998

(Application Serial No.)

(Filing Date)

60/112,855

December 16, 1998

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)  
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)  
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

**Leanne C. Price** Reg. No. 42,090

**Mariette A. Lapiz** Reg. No. 44,202

Send Correspondence to:

**Mariette A. Lapiz**

**FibroGen, Inc.**

**225 Gateway Blvd., South San Francisco, CA 94080**

Direct Telephone Calls to: *(name and telephone number)*

**Mariette A. Lapiz 650-866-7200**

6508667200

Full name of sole or first inventor <b>Bruce L. Riser</b>	
Sole or first inventor's signature	Date
Residence <b>Marshall, Michigan</b>	
Citizenship <b>U.S.</b>	
Post Office Address <b>409 Brewer Street</b>	
<b>Marshall, Michigan 49068</b>	

Full name of second inventor, if any <b>Mark DeNichilo</b>	
Second inventor's signature	Date
Residence <b>Daly City, California</b>	
Citizenship <b>Australia</b>	
Post Office Address <b>890 Campus Drive, #313</b>	
<b>Daly City, California 94015</b>	